**Universidade do Minho**
Escola de Engenharia

Anabela Alves Pinto

# Exploring the biomedical potential of a novel algae origin polysaccharide

Janeiro de 2012

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# Exploring the biomedical potential of a novel algae origin polysaccharide

Programa Doutoral em Engenharia Biomédica

Trabalho realizado sob a orientação do
**Professor Rui Luís Gonçalves dos Reis**
e do
**Doutor Rui Pedro Romero Amandi de Sousa**

Janeiro de 2012

*In loving memory of my mother*

*“I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.”*

*Marie Curie*

*“Any sufficiently advanced technology is indistinguishable from magic.”*

*Arthur C. Clarke*

## ACKNOWLEDGMENTS

As I stand here looking at a blank page with a blinking cursor without being able to wrap my mind around coherent words to write, I should say that this has to be the most difficult part of the thesis! There are several people that directly or indirectly contributed to the achievement of the goals defined in the beginning of my PhD. To them I want to express my sincere appreciation!

It was a complex but rewarding journey, which allowed me to learn and grow both as a scientist and as a person.
Doubts, mistakes, successes, failure...these are lessons learned and many times become beautiful memories!

My first words are to my supervisor, Prof. Rui Reis, who gave me the opportunity to be part of the 3B's Group. I've always admired his strength and perseverance that are the dynamic force behind the 3B's Group. He is *per se* an example and role model that pushes us harder and further, making us better scientists and allowing the placing of our group in a privileged position where it is recognized for the excellence of research performed on tissue engineering and regenerative medicine. I also want to acknowledge the patience and the advices that help me solve some of the obstacles that appeared along the way. Above all, thank you for giving me the tools and the freedom to do science.

To Rui Amandi, my co-supervisor, who guided me through my scientific work. I know I have this tendency to wander about things and sometimes loose the focus of my research. You said that this is because I'm a biologist and you were always able to bring me back on track. The first thing you asked me when I officially became your PhD student was for my thesis index. I found that a bit strange, but now I'm thankful for it; that index accompanied me during these 4 years and I must say that the main goals established back then remain valid! Thank you for your guidance and for all of our valuable discussions! You taught me about vision and entrepreneurship and told me to always keep the eye on the ball, to think outside the box and above all, 'don't crack under pressure'! Thank you!

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To everyone in the 3B's group that somehow contributed along the way. If I were to express my thankfulness to all the people that helped me along these years, I would certainly occupy more than a few lines...So I just say: thank you 3B's people, the past and the present ones! Thank you for the discussions, scientific or not, for the silences, the coffee breaks and the other breaks, the 'Tupperware' Club, for having an answer to my questions, for the lab help, for the fun (and stresses) we had...
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I also would like to acknowledge some people in the Department of Polymers Engineering (Guimarães), particularly Manuel Escourido, Maurício Malheiro and João Paulo. Thank you for all your help in the lab and with the equipments. You made my life so much easier!

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Mum, you have always been my rock, my driving force...You made me who I am and gave me the strength and the tools I needed to live my life to the fullest; you made me wish more and taught me that impossible is just a word! You are always in my heart, and you keep pushing me forward, when sometimes all I want to do is stop...This thesis is for you...You never gave up on me and I will never forget you...

Valério...what can I say? I love you so much that whatever I write here won't do it justice...these are just words... Thank you for your unconditional love, your kindness, your innocence! Thank you for supporting my decision of enrolling in a PhD and your ever funny question: 'Sis, please tell me again what is it you do?' Even though you don't fully understand the science that is the basis of my work, you are part of me and this passion of mine.

My final words are for my only and beloved Chico...You are the first in my heart...I don't know just how you could always manage to survive through all this, but you did! You were always there for me, waiting for me, giving me hope for tomorrow, making me a better person and a better scientist...you have always believed in me, even when I didn't...you put up with my silences, my absences every now and then...you understand them better than anyone. You brighten my life! You are my best, my truest friend, my love, my partner, my boyfriend, my husband!

There are so many people that take part on our life, either directly or indirectly...Some times they become truly important, other times they just pass by. I just wish I could thank all of them...

**EXPLORING THE BIOMEDICAL POTENTIAL OF A NOVEL ALGAE ORIGIN POLYSACCHARIDE**


## ABSTRACT

During the past decades, marine organisms have been the focus of considerable attention as potential sources of valuable materials. The sustainable exploitation and valorisation of natural marine resources constitutes a highly attractive and strategic platform for the development of novel biomaterials, with both economic and environmental benefits. In this context, algae are well known to synthesise large quantities of polysaccharides. In fact, algae are well established sources of these particularly interesting molecules, many of which are known for their applicability in the design of biomaterials. Among the three main divisions of macroalgae, green algae remain largely unexploited in the biomedical arena. While the demand for novel materials and technologies increases, so does the amount of research focused on these unexploited marine green algae including its unique polysaccharide ulvan. As research on this polysaccharide evolves towards the study of its potential application in a biomedical context, the definition of effective processing routes becomes rather crucial. The main objective of the herein described work is the development of biomedical applications for this green algae polysaccharide, with the additional advantage of adding value to a quite unexploited biomass. The innovative character lays on the application of traditional methodologies to extract and develop medical devices based on ulvan, a polysaccharide extracted from green algae, which presents a large, but yet untapped biomedical potential.

In order to attain the proposed objectives and in a first stage, a novel method of polysaccharide extraction from green algae has been designed and the resulting material characterized. The polysaccharide ulvan was obtained from the green algae *Ulva* by step extraction using hot aqueous solutions and precipitation with organic solvents. However and despite the care that is fundamental in the extraction of any natural material and the robustness of the employed methodologies, variability is frequently associated with natural extracts. Nevertheless, applying a simple extraction and purification methodology it was possible to extract a polysaccharide from green algae without significant changes in the overall structure of the polysaccharide. Extracted ulvan is a non-meltable semi-crystalline polysaccharide with high hygroscopic nature, highly sulphated and rich in ulvanobiuronic acid A and with minor amounts of ulvanobiuronic acid B. In general, ulvan is a high molecular weight polysaccharide, composed of rhamnose, glucuronic acid, xylose, iduronic acid and

glucose. Besides physicochemical categorization, toxicity data is regarded as a crucial and fundamental knowledge prior to any advances in the application development of natural derived polymers. In order to evaluate ulvan's potential cytotoxic behaviour, standard *in vitro* cytotoxicity screening assays were performed. Extracted ulvan demonstrated good results in terms of its non-cytotoxicity, comparable to a hyaluronic acid control, which suggests that ulvan can be considered as a non-toxic polysaccharide for the range of concentrations studied. Due to its unique structure and interesting inherent properties, ulvan can be considered as a versatile polymer for different applications in a biomedical context. In order to understand the limits of processability of ulvan extracted from green algae, different polymer processing methodologies were attempted. In a first approach, ulvan membranes envisioned towards applications as a medicated dressing were studied and revealed the potential as drug delivery devices for the envisaged applications as a wound medicated dressing. 3D porous scaffolds are widely used in tissue engineering contexts providing a three dimensional template for cell colonization and neo-tissue formation. In this regard, 3D porous structures based on ulvan were also produced by freeze drying and revealed a remarkable ability to uptake water and increased mechanical performance related with both polysaccharide concentration and cross-linking ratios. These structures are highly porous, interconnected, and undergo non-toxic degradation. At this point it is important to stress that ulvan is a polysaccharide soluble in water and prone to hydrolytic degradation, hence a modification step is necessary to render ulvan structures insoluble in water and stable at physiological conditions. Cross-linking with 1,4- butanediol diglycidyl ether (BDDE), a frequently used cross-linker for biomedical applications, was the chosen method to modify ulvan in order to produce both ulvan membranes and 3D porous structures. This cross-linking agent demonstrated to be successful in the cross-linking of this polysaccharide. Besides membranes and 3D porous structures, ulvan particles were developed by extrusion-dripping method and incorporated within a in a poly-D, L-lactic acid matrix in order to produce a novel scaffold with appropriate physicochemical and cytocompatible features to be used in bone engineering.

The knowledge gathered during this work encompass necessary and crucial steps to uncover the innovative biomedical potential of the marine derived polysaccharide ulvan, since the design of extraction methodologies from green algae to the study of diverse processing routes focused on the development of structures envisaged for specific biomedical applications.

**ESTUDO DO POTENCIAL BIOMÉDICO DE UM POLISSACÁRIDO EXTRAÍDO DE ALGAS VERDES**

## RESUMO

Organismos marinhos têm sido alvo de considerável atenção como potenciais fontes de materiais de elevado valor económico e tecnológico. A exploração sustentável e valorização de recursos marinhos constitui uma plataforma estratégica para o desenvolvimento de novos materiais, com benefícios tanto económicos como ambientais. Neste contexto, as algas sintetizam diferentes polissacáridos, muitos dos quais são conhecidos pela sua aplicabilidade no desenvolvimento de biomateriais para aplicação biomédica. Entre as principais divisões de macroalgas, as algas verdes permanecem largamente inexploradas nesta área. Enquanto a procura de novos materiais e tecnologias aumenta, o mesmo acontece com o estudo sobre algas verdes, em particular sobre o polissacárido ulvano. Há medida que o conhecimento sobre este polissacárido evolui no sentido do seu potencial de aplicação na área biomédica, a descrição de metodologias de processamento eficazes torna-se crucial. Assim, o principal objectivo do presente trabalho centra-se no desenvolvimento da aplicabilidade deste polissacarídeo extraído de algas verdes na área biomédica, com a vantagem suplementar de adicionar valor a um recurso marinho bastante inexplorado. O carácter inovador deste trabalho reside na aplicação de metodologias tradicionais para extrair e desenvolver dispositivos médicos com base em ulvano, que apresenta elevado, mas também insondado potencial biomédico.

Para atingir os objectivos aqui propostos e, numa primeira fase, um novo método de extracção de ulvano de algas verdes será desenvolvido e o material resultante será caracterizado. O polissacárido ulvano foi obtido a partir da alga verde *Ulva* por extracção com soluções aquosas a quente e precipitação com solventes orgânicos. No entanto, e apesar do cuidado que é fundamental na extracção de qualquer material de origem natural e da robustez das metodologias aplicadas, a variabilidade está frequentemente associada a extractos naturais. Ainda assim, a aplicação de metodologias de extracção e purificação simples permitiu a obtenção de um polissacárido com características interessantes sem alterações significativas na estrutura geral do polímero. O ulvano extraído é um polissacárido semi-cristalino, sem ponto de fusão detectado, com uma natureza higroscópica, rico em grupos sulfato e em ácido ulvanobiurónico A, com pequenas quantidades de ácido ulvanobiurónico B. Em geral, é um polissacárido de elevado peso molecular, constituído por ramnose, ácido glucurónico, xilose, ácido idurónico e glucose. Além do estudo da estrutura,

composição e propriedades físico-químicas, é necessário um estudo relativo à toxicidade deste polissacárido, considerado como um conhecimento fundamental anterior a qualquer avanço no seu desenvolvimento aplicativo. Neste sentido, ensaios padronizados de toxicidade foram efectuados e o ulvano demonstrou bons resultados em termos de citotoxicidade, comparável ao ácido hialurónico, o que sugere que pode ser considerado como não tóxico, nas condições estudadas. Devido à sua estrutura e propriedades, o ulvano poderá ser utilizado como um polímero versátil para diferentes aplicações num contexto biomédico. A fim de compreender os limites de processamento deste polissacárido, foram testadas diferentes metodologias de processamento de polímeros. Numa primeira abordagem, foram estudadas e confirmadas as propriedades filmogénicas deste polissacárido, com o objectivo de caracterizar a aplicabilidade de membranas de ulvano capazes de funcionar como sistemas de libertação de fármacos. Estruturas porosas são amplamente utilizadas em contextos de engenharia de tecidos fornecendo um modelo tridimensional para a colonização de células e formação de tecidos. Neste contexto, foram também produzidas estruturas porosas com base em ulvano, por liofilização, caracterizadas por uma morfologia porosa, com uma notável capacidade de absorção de água, propriedades mecânicas influenciadas tanto pela concentração do polissacárido como pela quantidade de agente reticulante utilizada e degradação não citotóxica. Neste ponto é importante realçar que o facto de este ser um polissacárido solúvel em água e susceptível a degradação por hidrólise, torna necessária uma etapa de modificação para tornar as estruturas produzidas à base de ulvano insolúveis em água e estáveis em condições fisiológicas. No presente trabalho, foi seleccionada a reticulação com um agente époxido como método passível de modificar o ulvano; este procedimento foi utilizado tanto na produção de membranas como de estruturas porosas aqui descritas. O uso deste agente, frequentemente utilizado em aplicações biomédicas, farmacêuticas ou cosméticas, demonstrou ser bem sucedido na modificação química por reticulação do ulvano. Além de membranas e estruturas porosas, foram desenvolvidas partículas à base de ulvano e incorporadas numa matriz de ácido poli-D, L-láctico, com o objectivo de desenvolver uma estrutura com características apropriadas para aplicações num contexto de engenharia de tecidos.

O conhecimento reunido durante a execução desta tese representa um passo necessário e fundamental para revelar a aplicabilidade biomédica do ulvano, desde a elaboração de metodologias de extração de algas verdes até ao estudo de diferentes métodos de processamento, focado no desenvolvimento de estruturas específicas previstas para aplicações biomédicas.

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## LIST OF ABBREVIATIONS

**B**

BCA – bicinchoninic acid

BDDE – 1,4-butanediol diglycidyl ether

**C**

C - carbon

CO – carbon monoxide

$CO_2$ – carbon dioxide

CP – crude protein

Cu – copper

**D**

$D_2O$ – deuterium oxide

DMA – dynamic mechanical analysis

DMEM – Dulbecco's modified Eagle's medium

DNA – deoxyribonucleic acid

dsDNA – double stranded DNA

DSC – differential scanning calorimetry

DX – dexamethasone

2D – two dimensional

3D – three dimensional

**E**

ECCC – European Collection of Cell Cultures

ECM – extracellular matrix

**F**

FBS – fetal bovine serum

FTIR – Fourier transform infrared spectroscopy

**G**

GAG – glycosaminoglycan

**H**

h – hours

H – hydrogen

$H_2O$ - water

HA – hyaluronic acid

**I**

IR – infrared

ISO – International Organization for Standardization

**L**

L929 – mouse C3H/Na connective tissue fibroblast-like cells

**M**

Mag. – magnification

Micro-CT (or µ-CT) – micro-computed tomography

Min - minutes

MTS – 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium

**N**

N – nitrogen

NADH – nicotinamide adenine dinucleotide

NADPH – nicotinamide adenine dinucleotide phosphate

NMR – nuclear magnetic resonance

**O**

O - oxygen

**P**

PBS – phosphate buffered saline

PDLLA – poly-(D, L-lactic) acid

**R**

rpm – revolution per minute

**S**

S – sulphate

Sec – seconds

SEM – scanning electron microscopy

st – stretching

**T**

t – time

T – temperature

TGA – Thermogravimetric analysis

**U**

UFC – *unité formant colonie* (colony-forming units)

UL – ulvan

UV-Vis – ultraviolet-visible spectrophotometry

**V**

Vol. – volume

v/v – volume in volume

**W**

w/w – weight in weight

w/v – weight in volume

**X**

XL – cross-linker

XRD – X-ray diffraction

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## SHORT *CURRICULUM VITAE*

Anabela Alves Pinto was born on May 6, 1981, in Vila Nova de Gaia, Portugal. She is currently a PhD student at the 3B's Research Group (Biomaterials, Biodegradables and Biomimetics), at the University of Minho, Braga, Portugal. Her work is supervised by Prof. Rui L. Reis, also the Director of the 3B's Research Group, and Rui Amandi de Sousa, senior researcher at the 3B's Research Group and Chief Executive Officer of Stemmatters, an academic spin-off of the 3B's Research Group.

She has a degree in Biology (scientific branch) from the University of Aveiro, which she finished with a final graduation mark of 15 (0-20 scale). In the final stage of her degree, she was a research trainee at Ecotoxicology Research Group of the University of Aveiro working in bivalves genotoxicity induced by xenobiotics under the supervision of Prof. Maria Ana Dias Monteiro Santos, University of Aveiro, Portugal, and Dr. Manuel Sobral, IPIMAR, Aveiro, Portugal, where she received a traineeship mark of 19 (0-20 scale).
Her work in ecotoxicology continued in CIIMAR, Porto, Portugal, where she collaborated on the study of toxic effects induced by environmental contaminants on natural eel populations, under the supervision of Prof. Lúcia Guilhermino.

In October, 2007, she formally started her PhD work at the 3B's Research Group with a main focus on ulvan and its applicability on a biomedical arena, which was further supported by a grant received from the Portuguese Foundation for Science and Technology (FCT) in January, 2008.

During the course of her PhD, in September 2008, she has received an award (second place) in Young Persons' World Lecture Competition, organized by The Institute of Materials, Minerals and Mining (IOM3), in Florida, USA. Since then, she was responsible for the organization of the Portuguese Young Persons' Lecture Competition, yield in the 3B's Research Group, in order to select a Portuguese candidate to represent Portugal in the World Lecture Competition.

As a researcher in the 3B's Research Group, she has been involved in the writing, preparation, and execution of several grant proposals both at National and European levels.

As a result of her research work, she attended several important international meetings in her field of research mostly with oral communications. Presently, she is author of 10 research papers (6 as first author), 1 book chapter, 20 communications (oral and poster), and 1 patent.

## LIST OF PUBLICATIONS

*1. Publications resulting from the work performed during this PhD*

**International journals with referee**

**Alves A**, Caridade SG, Mano JF, Sousa RA and Reis RL, Extraction and physico-chemical characterization of a versatile biodegradable polysaccharide obtained from green algae. 2010. Carbohydrate Research, 345: 2194-2200

**Alves A**, Pinho ED, Neves NM, Sousa RA and Reis RL, Processing ulvan into 2D structures: cross-linked ulvan membranes as new biomaterials for drug delivery applications. 2012. International Journal of Pharmaceutics, doi: 10.1016/j.ijpharm.2012.01.021.

**Alves A**, Sousa RA and Reis RL, A practical perspective on ulvan extracted from green algae. 2012. *Submitted*.

**Alves A**, Sousa RA and Reis RL, *In vitro* cytotoxicity assessment of ulvan, a polysaccharide extracted from green algae. 2012. *Submitted*.

**Alves A**, Sousa RA and Reis RL, Processing of degradable ulvan 3D porous structures for biomedical applications. 2012. *Submitted*.

**Alves A**, Duarte ARC, Mano JF, Sousa RA and Reis RL, PDLLA enriched with ulvan particles as a novel 3D porous scaffold targeted for bone engineering. 2012. *Submitted*.

**Procedings of conferences published in international journals**

**Alves A**, Pinho ED, Neves NM, Mano JF, Sousa RA and Reis RL, 2008, Ulvan: a new natural derived biomaterial obtained from renewable marine resources, Tissue Engineering: Part A, 14 : 793.

**Alves A**, Duarte ARC, Mano JF, Sousa RA and Reis RL, 2011, Subcritical sintering of an aliphatic polyester enriched with ulvan particles loaded with a bioactive agent, International Journal of Artificial Organs, 34, 8, 703.

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**Alves A**, Sousa RA and Reis RL, A novel polysaccharide for biomedical applications: a bottom up approach to establish ulvan as a biomaterial, Society for Biomaterials 2010 Annual Meeting and Exposition, Seattle, United States of America, April 2010 (*Oral Presentation*)

**Alves A**, Sousa RA and Reis RL, Green algae as source of a polysaccharide – ulvan – suitable for biomedical applications, 2nd IBB (Institute for Biotechnology and Bioengineering) Scientific Meeting, Braga, Portugal, October 2010 (*Poster Presentation*)

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*2. Publications resulting from collaborative work within and outside the 3B's*

**International journals with referee**

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Silva TH, **Alves A**, Ferreira BM, Oliveira JM, Reys L, Ferreira RJF, Sousa RA, Silva SS, Mano JF, Reis RL, Materials of marine origin: a review on polymers and ceramics of biomedical interest. 2012. International Materials Reviews, *Accepted for publication*.

**Conferences and meetings**

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Silva TH, **Alves A**, Ferreira BM, Gomes S, Sousa RA, Mano JF, Neves NM and Reis RL, Methodologies for the isolation of several marine origin polymers and ceramics with high potential for valorisation, PROTEUS Project Public Meeting, Vigo, Spain, December 2007 (*Oral Presentation*)

Silva TH, **Alves A**, Ferreira BM, Silva SS, Sousa RA, Mano JF, Neves NM and Reis RL, Marine derived materials on the development of biomedical applications, 1st IBB (Institute for Biotechnology and Bioengineering) Scientific Meeting, Faro, Portugal, May 2009 (*Poster Presentation*)

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## INTRODUCTION TO THE THESIS FORMAT

The present thesis is organized in 5 sections reflecting the main goals defined for the thesis and the nature of the performed experiments. These sections are divided into chapters which are based in papers already published or submitted for publication in international journals, which are identified at the front page of each chapter.

As a preamble to the work, a concise introduction about ulvan, its properties and the present state of the art is presented (**Section I, Chapter 1**). Following this introductory note, a description of the chosen materials and methods is presented in order to facilitate the understanding of the herein presented work and its principles (**Section II, Chapter 2**). Although the following chapters are accompanied by specific materials and methods sections, Chapter 2 intends to complement these sections with additional and relevant information about the materials and methodologies employed in the present research work.

After gathering knowledge about ulvan and its peculiarities and assembling the tools to work this polysaccharide, the main objective of the herein described work was the study of potential development of biomedical applications based on ulvan. A simple way to contextualize the complexity and diversity of work developed is to define this thesis as the application of fundamental research principles for the development of basic and applied knowledge towards translational science of ulvan. An explanatory scheme of the general overview and objectives of the present thesis is depicted in Figure 1.

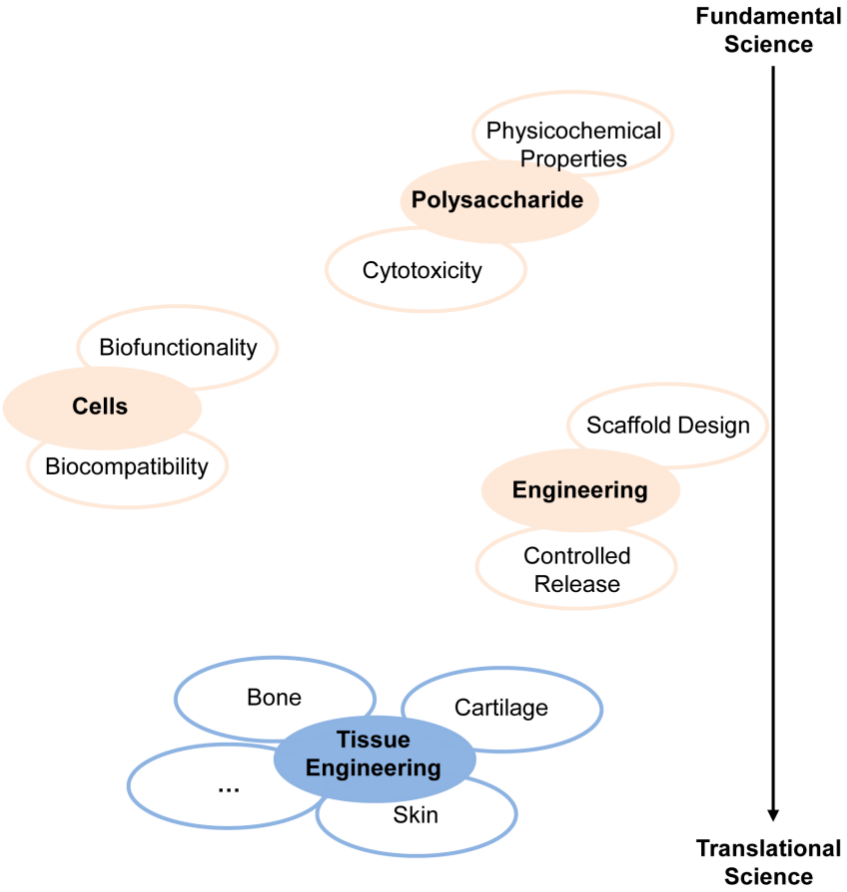


**Figure 1.** Representative scheme of the thesis work and format. (Adapted from Clemens van Blitterswijk & Jerome Sohier, in Tissue Engineering, Academic Press, Elsevier, UK, 2008)

In this context, traditional methodologies were applied to extract and develop medical devices based on ulvan, a polysaccharide extracted from green algae, which presents a large, but yet untapped biomedical potential.

In a first experimental stage, the polysaccharide itself was explored, namely its extraction from green algae and subsequent characterization (**Section III, Chapter 3 and 4**).

The polysaccharide ulvan was obtained from green algae *Ulva* by step extraction using hot aqueous solutions and precipitation with organic solvents (Chapter 3). The use of simple extraction and purification methodologies allowed the extraction of a polysaccharide from green algae. At this point, it is important to stress that a concise study on the composition of ulvan extracted from green algae and the effect of purification methodologies on the final extract was performed within the scope of the present PhD work plan, in cooperation with an external research laboratory. However,

this study was not included in the present compilation of results. It resulted in a a paper that can be consulted in addition to this thesis for a better understanding of applied extraction methodologies and composition of the extracted ulvan (Costa C, *Alves A*, Pinto P, Sousa RA, Silva E, Reis RL, Rodrigues A, Characterization of ulvan extracts to assess the effect of different steps in the extraction procedure. 2012. Carbohydrate Polymers, doi:10.1016/j.carbpol.2011.12.041).

Following the design of a novel extraction procedure, additional characterization was performed by focussing on particular characteristics regarded as important for future studies based on this polysaccharide (Chapter 3). Besides physicochemical characterization, toxicity data is a critical knowledge prior to any advances in research of natural derived polymers, especially if biomedical applications are intended. Hence, standard cytotoxicity tests were used for investigating the toxicity of ulvan extracts. (Chapter 4).

The structure and properties of ulvan make this biodegradable polymer an interesting option for different biomedical applications. In this context, and in order to understand the processing versatility of ulvan, different processing methodologies were attempted (**Section IV, Chapter 5, 6 and 7**). Within the scope of this thesis, three approaches were followed. In the first study, ulvan membranes and its properties were evaluated towards applications as a medicated dressing (Chapter 5). In another study, 3D porous structures based on ulvan were produced by freeze drying and also characterized (Chapter 6). In addition to membranes and 3D porous structures based on ulvan, ulvan capsules were also produced and incorporated within a in a Poly-DL-Lactic acid matrix in order to produce a novel and functional scaffold with appropriate physicochemical and cytocompatible properties to be used in tissue engineering (Chapter 7).

The knowledge gathered during this work, including those related with polysaccharide extraction, characterization and processing into diverse type of structures constitutes an important step to elucidate on the biomedical potential of this marine derived polysaccharide. The concluding remarks are presented in the final chapter of this thesis (**Section V, Chapter 8**).

# SECTION I – GENERAL INTRODUCTION

# Chapter 1

# A practical perspective on ulvan extracted from green algae

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This chapter is based on the following publication: Alves A, Sousa RA and Reis RL, A practical perspective on ulvan extracted from green algae. 2012. *Submitted*.

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## 1. Introduction

World's oceans are a rich environment containing over 300,000 invertebrates and algal species (as cited in Pomponi, S. A., 1999).[1] These species survive and live within complex communities and in close association with other organisms. This diversity of living systems and habitats define the basis of the wide variety of chemical classes typical of marine derived molecules. Some organisms withdraw their rich chemistry from dietary sources, whilst others synthesize these compounds *de novo*.[2] Molecular diversity represents a vast and valuable chemical library, including saccharides, pigments, phenols or peptides, among others, which together are estimated to possess a potential market value of several billion dollars.[1]

In this scenario, marine algae, rich in different compounds of interest, are used in several industrial application contexts totalizing a consumption total of 3.5 million tons of algae per year worldwide (as cited in Michel, C. and McFarlane, G. T, 1996).[3] Applications include for example food and the industrial use of hydrocolloids, like alginate, agar and carrageenan.[3] The importance of these organisms and their constituents to Mankind has justified intense research work; however the full potential of algae molecules is yet to be unveiled.

Marine algae can be divided in three main groups – red (Rhodophyceae), brown (Phaeophyceae) and green (Chlorophyceae) – classified on the basis of their photosynthetic pigments.[4] These algae also differ on the type of storage material and cell wall polysaccharides.[4-5] Brown algae synthesize laminaran as storage material while floridean starch (glycogen) and starch are found in red and green algae, respectively.[4-5] All algae possess varying amounts of cellulose in their cell walls.[5] However, the main polysaccharide found in the cell wall varies from alginates and fucans in brown algae to different sulphated polysaccharides, xylans and mannans found in red and green algae.[5-6] Algae polysaccharides are strikingly different of those found in higher plants, especially on what concerns to the presence of sulphate groups and unusual sugar residues, high content of ionic groups, high water solubility and unique rheological properties.[3, 7-8] This results in a range of distinctive characteristics and is the basis of their widespread industrial applicability. A large fraction of the world commercially exploitated polysaccharides are of marine origin, namely alginate, agar and carrageenan.[3, 7, 9] Within this context, green algae are still rather unexploitaded. Although they have been used in food for many centuries, it was the discovery of an important constituent of green algae, ulvan, which dramatically increased interest in these algae.

In this regard, focused research on ulvan's characteristics and applicability is required in order to grasp the full range of its capabilities and boost the industrial interest in green algae.

## 2. Historical outline

Classification of alga hasn't always been an easy task. Complexity is enhanced when recent genetic studies reveal that *Chloropelta*, *Enteromorpha* and *Ulva* in fact belong to the same genera.[10] In general, Chloropyceae consist of an uni- or pluri-cellular plant body, containing bright-green chromatophores, producing starch and with a cellulosic cell wall, that can be solid or mucilaginous.[4, 11]

As the work with brown and red macroalga evolved to a well established field of knowledge, the interest in green algae, and especially its polysaccharides had a late and bumpy start. An interesting and complex polysaccharide composed of sulphate ester, uronic acids and xylose, rhamnose and glucose residues, was identified in the early 40-50's. This pioneer work was however interrupted for several years due to the Second World War.[12] In these early studies, Brading and co-workers[12] already identified a possible structure present in this sulphated polysaccharide as –CH(OH)-CH(O-$SO_3$Na)-CH(OH)-. However, no evidence was found on what concerns the true position of the sulphate groups on the polysaccharide structure, although it seemed possible that they should be linked to glucose or rhamnose.[12] This was also one of the first times rhamnose was identified as being part of an algal polysaccharide. In 1962, McKinnell and co-workers[13] found evidences that the sulphate groups were in fact linked to rhamnose. Ten years later, Abdel-Fattah and Edrees[14] concluded on the heteropolysaccharidic nature of this polysaccharide, composed of particular entities of oligosaccharides of rhamnose and uronic acids composing its backbone, intercalated with sequences involving glucose, arabinose and xylose. The particularly acidic nature of the polysaccharide was assigned to the presence of glucuronic acids.[14] In these early years, researchers were struggling with the identification and characterization of this unusual and complex polysaccharide. Extracts were obtained from different green algae genus and species and were characterized and compared between each other. Despite the fact that different researchers were extracting a polysaccharide from different genus and species of green algae, through diverse extraction methodologies, they all came to similar conclusions and agreed that the water soluble polysaccharide from green algae was mainly composed of uronic acid and sulphated rhamnose with residues of diverse sugars, like xylose and glucose. [9, 12-18] It was only in 1993 that the

designation ‘ulvan’ was proposed.[9] It is now generally accepted that ulvan designates a class of sulphated polysaccharides mainly composed of glucuronic acid and sulphated rhamnose.[18] The important discovery that iduronic acid was a constituent of ulvan’s backbone was only made in 1997.[19]

There is still a long way to go until the true nature of this polysaccharide is unravelled. However, in the last decade research on ulvan has evolved as the knowledge on its occurrence and physiological function, chemical composition, polysaccharide conformation and properties has improved. This knowledge is now driving research a step forward towards the application development of this polysaccharide.

## 3. Occurrence and physiological function

Marine algae are simpler organisms compared to land plants; however, they produce larger quantities of polysaccharides.[20] These polysaccharides differ from the ones produced by land plants mostly due to the presence of uronic acid and/or sulphate ester residues.[8, 20] In fact, algae synthesise sulphated polysaccharides as key constituents of the extracellular matrix.[8]

In general, green algae are composed of: ~11% protein, ~36% carbohydrates, ~53% ashes and are rich in minerals like calcium, iron, phosphorous and chloride.[21] Carbohydrates include cell-wall water soluble sulphated ulvan, alkali-soluble hemicellulosic β(1,4)-D-glucuronan and β(1,4)-D-glucoxylan and amorphous α-cellulose with xylose residues.[18, 22-25]

*Ulva* sp. possess a characteristic blade-shaped frond, two-cell thick, with no tissue differentiation.[25] However, their cellular walls are well organized in layers and rich in polysaccharides.[8, 25] Within this scheme, ulvan is mostly homogeneously distributed throughout the frond being more predominant within the intercellular space and in the fibrillar wall.[25] Within this cell wall moiety, it is suggested that ulvan may be arranged in a bead-like structure, stabilized by cell wall proteins or strong physical interactions.[26] It is well known that boron is accumulated in algae, in the form of boric acid in green algae, and it was suggested that complexes with carbohydrates.[27] Given that ulvan is able to gel in the presence of boric acid, in a reaction mechanism mediated by calcium ions[9], it is easy to hypothesize that this polysaccharide may be present in green algae cell wall in the form of a gel, cross-linked by boron ions.

As far as ulvan’s physiological function within the cell wall goes, literature is rich in hypothesis.[8, 19-20, 25, 28-31] In general, the presence of sulphated polysaccharides can be

associated with the organism's adaptation to ionic environments; this is true for marine plants and algae (seawater) as well as for vertebrates (physiological saline serum).[8] In this scenario, ulvan, as part of the cell wall of green algae, would possess osmotic functions, including a role on ionic balance or on the prevention of algae desiccation, due to their highly hygroscopic nature.[20, 31] On the other hand, ulvan is resistant to bacterial degradation and inhibits the activity of cellulase, which indicates a protective role towards cell wall amorphous α-cellulose, protecting it from bacterial attack.[25, 28, 30] This protective function is also related with the fact that ulvan is associated with the low porosity of green algae.[25, 30] Furthermore, ionic polysaccharides may be involved in mechanical regulation, spore release and adhesivity.[8, 29] The presence of acidic moieties in ulvan's polysaccharidic chain, particularly glucuronic and iduronic acid, may indicate that this polysaccharide is also involved in cell wall cohesion, in a parallelism with mammalian glycosaminoglycans rich in uronic acids.[19, 30]

## 4. Extraction of ulvan from green algae

This section is intended to provide guidance to those interested in extraction of natural products, especially from green algae. Success in working with nature derived materials is very well summarized by Cannell and co-workers[2] "one should always keep an open mind, expect the unexpected, use as many methods for purification as possible, and save all fractions."

The overall procedure to obtain polysaccharides from green algae can be divided in several different steps:

- Selection, collection, and identification of the raw material;
- Algae stabilization and grinding;
- Extraction and purification;
- Precipitation;
- Drying.

A representation summarizing the general procedure to extract ulvan from green algae is illustrated in Figure 1.1.

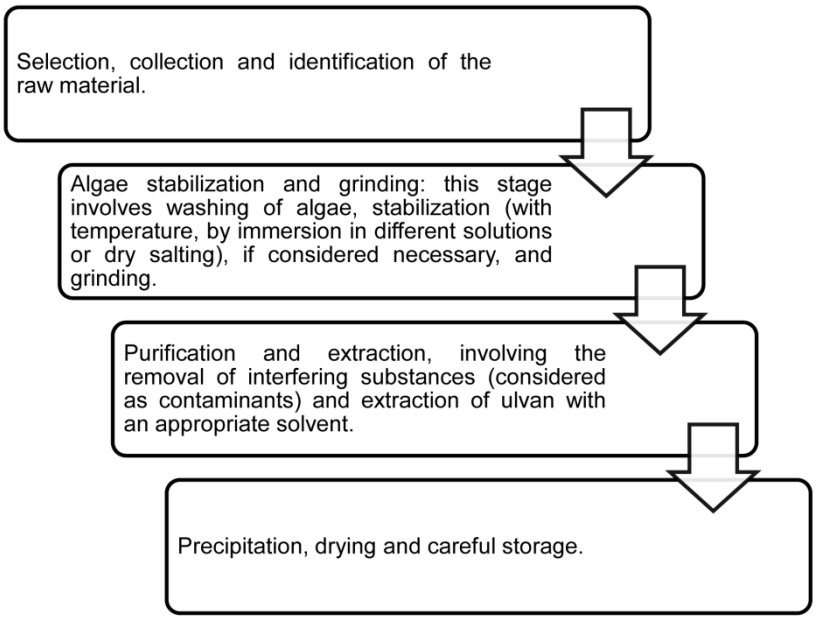


**Figure 1. 1.** Scheme for the extraction of ulvan from green algae.

When working with nature derived materials, one should always recognize and consider the inherent variability of extracted molecules from nature origin sources.[32] This can be due to varying organism species, extraction methodologies, seasonality, habitat or life cycle.[9, 14, 33-36] One practical example is the variability of the obtained polysaccharide related with source reported by Lahaye and Axelos[9], when comparing the rheological behaviour of a polysaccharide obtained from proliferating *Ulva* with one obtained from *Ulva lactuca*.

### *4.1. Selection, collection, and identification of the raw material*

The first step in extraction of algae derived materials involves the selection, collection and identification of the raw material. During these initial stages, one should try to answer some questions:

- What is the rational for the extraction of a particular polysaccharide?
- What is the most accessible source of algae available suitable for the extraction of such polysaccharide?
- Can a compromise between yield and purity be obtained by appropriately selecting the algae species?
- Is it possible to have access to large quantities of the selected algae species?

The answer to these questions defines the basis of any future extraction work and allows the establishment of good criteria for potential industrial applications of ulvan.

In order to obtain the selected algal raw material, it is possible to collect it *in loco*, culture it or obtain it through specialized enterprises (Table 1.1). To address this issue one should keep in mind that besides source dependence, cross-contamination by other organisms, habitat, geography and seasonality are relevant factors that influence and further complicate the work with marine natural products.[2, 37] Furthermore, among the biggest challenges when working with algae is its identification, which many times can only be done correctly by an expert algologist.[2, 37]

Algae can be obtained by harvesting natural seaweed beds or by algaculture.[3] The availability of green algae allows a sustainable exploitation of this resource.[1] They are many times involved in algal blooms with detrimental health and socio-economic impact.[9, 38-39] These algal blooms occur in nutrient rich waters, stimulated by human activity in coastal regions.[9, 38-39] Partial or total utilization of these algae and particularly of its polysaccharide ulvan would alleviate the effect of accumulation and elimination of these deleterious biomasses. Furthermore, it is easily cultured in (semi-) artificial conditions, both in-the-sea and land-based, alone or in integrated systems.[4, 40-42] These facts make green algae easily accessible to research work and application development.

**Table 1. 1.** Examples of enterprises specialised in the commercialization of algae.

| Company | Location | Algae | Culture System | Website |
|---|---|---|---|---|
| Sinaloa Seafields International Inc. | California, USA | *Ulva* sp. | Algae culture in non-arable land with sea water irrigation | http://www.sinaloa-seafields.com/home/ |
| Atlantic Mariculture Ltd. | Grand Manan Island, Canada | *Ulva* sp. | Local harvest of native sea vegetables | http://organicdulse.com/ |
| Setalg | Pleubian, France | *Ulva lactuca* | Harvest on algae fields | http://www.setalg.com/ |
| Mariculture Technologies International, Inc. | Florida, USA | *Ulva* sp. | Local harvest (wild collected) | http://www.mariculturetechnology.com/ |

### *4.2. Algae stabilization and grinding*

The first extractions of polysaccharides from green algae usually started with washing of algae prior to thermal drying.[5] Nowadays, relevant advances in seaweed and hydrocolloid industries reveal other alternatives to algae stabilization treatments prior to polysaccharide extraction. Robic and co-workers[43] have studied the effect of different

green algae pre-treatments, prior to polysaccharide extraction, including freezing, drying methods, brining and dry salting. These different algae stabilization treatments demonstrate a marked effect on the final yield of extraction and on ulvan's physicochemical traits, including molecular weight and viscosity. Higher yield is calculated for algae brined for seven weeks; however, higher molecular weight and viscosity is calculated for frozen and freeze dried algae.[43] These results emphasise the need for a concise evaluation of the final application of the extracted polysaccharide and the expected cost and efficiency of the extraction procedure. Furthermore, it is important to note the effect of these pre-treatments on the chemical composition of the polysaccharide, in particular due to polysaccharide modification or even degradation.[43]

### *4.3. Extraction and purification*

Before and after extracting the polysaccharide *per se*, one must be aware of interfering substances that can be co-extracted with the molecule of interest, and of other contaminants introduced during extraction, etc. These substances and contaminants can either affect purity of the extract or interfere with biological responses towards the polysaccharide.[2]

Removal of photosynthetic pigments (decoloring) and lipids (defating) may be considered the as the first steps of purification. Decoloring and defating can be achieved by different methods, including supercritical extraction, soxhlet extraction or simple immersion in organic solvent.[2, 12-13, 15-17, 44] Being considered a green technology, the use of supercritical fluids avoids or reduces the use of organic solvents. However, this is an expensive technology and the use of organic solvents is still more effective and widely used, both for decoloring and defating.[45] In this regard, soxhlet extraction is an old but effective technique.[46] Different organic solvents may be used to remove pigments, including acetone[12, 15-16, 47], ethanol[13, 47-49], methanol[17, 47], petroleum ether[32, 50] or chloroform[32]. However, McKinnell and co-workers[13] found that the best solvent to remove colouring matter from green algae is 85 % aqueous ethanol. Kitada and co-workers[45] also found ethanol a more effective solvent to extract pigments, when compared to acetone; furthermore and considering safety issues, ethanol presents lower toxicity compared to acetone. On the other hand, treatment of algae with ethanol prior to polysaccharide extraction increases the total sugar content of the extract and decreases the presence of minor sugars and glucose on the final polysaccharide extract.[26, 51]

Effective extraction of a natural origin polysaccharide is mainly dependent on its solubility, stability, and functional-group considerations.[2] Being a water soluble

polysaccharide, ulvan can be effectively extracted with water.[18, 49] Some studies report that hot water extraction results in good extraction yields.[13, 26, 35] Hot water disrupts the cell walls, allowing easier access to its content.[52] High temperatures, in the range of 80-90ºC, allow the extraction of higher molecular weight polysaccharides.[35] However, above 100ºC, lower viscosity is detected, when compared with ulvan extracted at 80-90ºC[35], which may reflect some instability of the polysaccharide extracted in this range of temperature.[37] Ulvan can also be successfully extracted with sodium carbonate solution[12], calcium chelating agents, such as ammonium oxalate or ethylenediaminetetraacetic acid (EDTA) [14, 26, 36, 47] or acidic solutions.[14, 17, 26, 30, 36] The use of calcium chelating agents facilitates ulvan extraction by means of sequestering calcium ions and disrupting chemical bonds formed by ulvan in the presence of calcium ions within the cell wall of green algae.[14, 26] On the other hand, decrease of pH of the extraction media will destabilize aggregates of ulvan within the cell wall, increasing extraction yield.[26] The use of different solvents to extract ulvan will result in extracts with varying physicochemical and biological properties.[14, 26, 36, 44, 47] In this regard, it is important to define the objective of the extraction and the final properties expected from the resulting polysaccharide.

Being a cell wall material, common contaminants of this polysaccharide are amino acids and peptides.[12-13, 53] In fact, direct extraction with hot aqueous solutions will result in an extract mixture of polysaccharides, proteins, polyphenols and pigments.[52] Methods to remove nitrogenous materials include enzymatic treatment with proteinase K[49] or precipitation with trichloroacetic acid.[13] Besides nitrogenous material, McKinnell and co-workers found evidences of contamination by starch-like materials in hot water extracts.[13] However, this starch can be effectively removed with salivary α-amylase.[13, 16, 54-55] Smaller molecular weight contaminants, including the ones responsible for odour and also colour can be removed with activated charcoal[2, 17, 49]; hydrogen peroxide can also be used as a decoloring agent.[56] Further purification may be achieved by other methods, including dialysis (salting-out) or even through final precipitation.[51]

### *4.4. Precipitation*

In general, water soluble polysaccharides are removed from solution by precipitation with organic solvents. In the particular case of ulvan, it precipitates from aqueous solutions with ethanol or acetone, usually four volumes of the chosen organic solvent.[17, 44, 49] Ethanol precipitation allows the separation of the polysaccharide from low molecular ethanol-soluble compounds[52] and the removal of some pigments[6]; thus it is considered as a purifying step as well.[51, 57-58]

### *4.5. Drying*

Extracted polysaccharide can be dried by different methods. However, Jani and co-workers[32] highlight the need to apply low temperature or vacuum on drying, in order to avoid degradation of the polysaccharide.

### *4.6. Yield*

Cell wall carbohydrate content of green algae ranges from 38 to 54% and ulvan content may vary between 18 to 29%.[37, 59] Yield of extraction of ulvan varies between 1.2 to 27.5% and the maximum extraction efficiency is in the range of 70%. [13-14, 16-18, 26, 31, 34-36, 43-44, 47, 49-50, 60]

In general, a decrease in algae particle size will increase the yield of extraction.[26] This is also influenced by factors including algae pre-treatments, solvent used to extract the polysaccharide *per se*, number, duration and temperature of extractions, purification methodologies, algae species and seasonality.[14, 16, 26, 34-37, 43-44]

### *4.7. Storage*

As polysaccharides, and ulvan in particular, are hydrophilic and readily uptake water from the atmosphere, it is recommended to store the extracted polysaccharide in a dry environment, ideally on desiccators, to avoid moisture uptake and degradation.[32, 49]

## 5. Chemical structure and conformation

Ulvan has been identified as being a sulphated single polydisperse heteropolysaccharide composed of variable amounts of uronic acids, including glucuronic and iduronic acids alternating with neutral sugar moieties, such as rhamnose, xylose and glucose residues, connected by α- and β-1→4 bonds.[12, 16, 19, 23-24, 30, 53, 61] This polysaccharide accounts for 18-29% of the carbohydrate fraction of green algae.[59] Important insights on the structure of this polysaccharide were provided by McKinnell and co-workers.[13] They found evidences that the sulphate groups were in fact linked to rhamnose, possibly in position 2; they suggested possible branching of the heteropolysaccharide and uronic acid residues are pointed as possible end groups, being these attached to position 4 in rhamnose.[13] Later on, it was proposed that sulphate groups may be linked to C-2 or C-3 in rhamnose and there are evidences of the presence of some sulphated xylose, with sulphate occurring in position 2. [16, 18, 22] In 1997, an important work of Quemener and co-workers[19] exposed the presence of iduronic acid within ulvan's backbone. In general, green algae synthesise the same

sulphated polysaccharide. However, the particular amount of each monosaccharide residue or the arrangement of the polysaccharide may vary with different factors, including methodology of extraction[16, 44, 54], geographical distribution or species[44, 53, 61], maturity, environmental condition and seasonality[34, 62], resulting in varying ulvan structures.[37] This variability influences the establishment of accurate ulvan sugar composition, complicated by the difficulty to determine the presence of different characteristic sugars. This is due to the resistance of aldobiuronic acid to acid hydrolysis and due to the labile nature of iduronic acid, easily degraded by the strong acidic conditions needed to hydrolyse ulvan.[15, 37, 51, 63] In order to minimize these effects, innovative methods that combine mild acid hydrolysis with enzymatic degradation have been developed and allow an accurate insight into ulvan's sugar composition and the authentication of the presence of iduronic acid within its backbone.[19, 55]

It is now generally agreed[3, 23, 53, 61] that the backbone of ulvan is mostly composed of α- and β-(1→4)- linked sugar residues, namely of α-1,4- and α-1,2,4- linked L-rhamnose 3-sulphate, with branching at O-2 of rhamnose, β-1,4- and terminally-linked D-glucuronic acid and β-1,4-linked D-xylose, partially sulphated on O-2. The major structural units are the β-D-glucuronosyluronic acid-(1,4)-L-rhamnose 3-sulphate dimer (β-D-Glc*p*A-(1→4)-L-Rha*p* 3-sulphate) and α-L-Ido*p*A-(1→4)-α-L-Rha*p* 3-sulphate, also known as ulvanobiuronic acid A and B, respectively.[19, 23-24, 30, 53, 61, 64] The main difference between these aldobiuronic acids is the presence of glucuronic acid in A, which is replaced by iduronic acid in B.[30] Variations in these main motifs can also be encountered.[37, 61]

One remarkable feature of ulvan is the presence of rare sugars within its backbone, namely sulphated rhamnose and iduronic acid. Rhamnose is an unusual sugar, usually found in bacteria and rare in animals, and branching of O-2 of 1,4-linked α-L-rhamnose residue was found only on an exopolysaccharide synthesised by the *Arthrobacter* sp. bacterium.[23] The presence of iduronic acid in ulvan's polysaccharidic chain represent another striking characteristic, as this sugar residue has never been identified in algal polysaccharides.[19]

## 6. Enzymatic degradation

Degradation of polysaccharides occurs through a sequence of abiotic and/or biological reactions. In order to get a sense on the biodegradation of ulvan, knowledge of its enzymatic cleavage becomes essential. In general, polysaccharide enzymatic

degradation is carried by polysaccharide hydrolases or polysaccharide lyases.[57] Polysaccharide lyases, in particular, degrade the polysaccharidic chain by β-elimination reactions and are usually isolated from bacteria, algae, gastropods and fungi.[57, 65] Despite this knowledge, only a few enzymes with ulvan lyase activity were identified so far and are summarized in Table 1.2. Generally, the discovery and isolation of novel polysaccharide enzymes is based on the need of enzymatic hydrolysis of polysaccharides as a tool for better understanding its structure.[51]

In this regard and in order to try to elucidate the chemical structure of ulvan and understand the enzymatic degradation of this polysaccharide, an *endo*-ulvan lyase has been isolated from a marine Gram-negative bacterium.[24] These bacteria were selected from slurry rich in decomposing green algae *Ulva* and their ulvanolytic activity requires the presence of calcium chloride and demonstrates optimum activity at pH 9.[24] Depolymerisation of ulvan is an endo-molecular process and ulvanobiouronic acid A motif (β-D-Glc*p*A-(1→4)-L-Rha*p* 3-sulphate) is cleaved resulting in different saccharides with 4-deoxy-L-*threo*-hex-4-enopyranosyluronic acid at the reducing end.[24, 66] The isolation of this lyase permitted new insights on ulvan's chemical composition.

The same objective was on the base of the work of Quemener and co-workers[19] which were able to purify a β-glucuronidase from the snail *Helix pomatia* and used it to optimize studies on the different sugar composition of ulvans from various sources. Within their work, a chemo-enzymatic method was developed to optimize the release of stable monosaccharides after ulvan's degradation and β-glucuronidase was successively used to hydrolyse the resistant disaccharide aldobiuronic acid.

The work of El Boutachfaiti and co-workers[67] resulted on the successful isolation of an ulvan-lyase from a bacterium from the genus *Ochrobactrum*. This particular enzyme cleaves the osidic bond bridging ulvanobiuronic acid A and ulvanobiuronic B, through a β–elimination reaction.[67]

Another enzyme with ulvan lyase activity was isolated from a fungus prevalent in soils, *Trichoderma* sp., and it was identified as a glucuronan lyase. It is dependent on the presence of some ions, including calcium and magnesium, and is sensitive to temperature. Ulvan's degradation by this fungal glucuronan lyase originates low molecular weight polysaccharides.[68]

Due to the presence of uronic acids within ulvan's backbone, many authors[19, 53, 69] have compared it with mammalian glycosaminoglycans, like chondroitin sulphate. In this sense, and in a biomedical application context, one can infer about the enzymatic

susceptibility of ulvan towards naturally occurring mammalian enzymes that degrade this type of molecules into sulphate residues and monosaccharides, such as β-glucuronidase, hyaluronidase and aryl sulphate.[70] Given the evolution of the knowledge of ulvan so far, it should be expected that some of the research focus should now shift towards the understanding of ulvan's enzymatic degradation and the nature of the released products. These studies are especially important when applications for instance in medicine, food or pharmaceutics are envisaged. In a particular example, within a context of tissue engineering and regenerative medicine, biodegradability (hydrolytic and/or enzymatic) in physiological conditions is one of the key and crucial properties of biomaterials.[71]

**Table 1. 2.** Summary of identified enzymes with ulvanolytic activity, compiled from the references [19, 24, 66-68].

| Enzyme | Origin | Activity | References |
|---|---|---|---|
| *Endo*-ulvan lyase | Marine Gram-negative bacterium | Cleavage of (1→4) linkage between rhamnose 3-sulphate and glucuronic acid | 24, 66 |
| β-Glucuronidase | Land Snail | Cleavage of (1→4) linkage between rhamnose 3-sulphate and uronic acid | 19 |
| Ulvan lyase | Bacteria | Cleavage of the osidic bond between ulvanobiuronic acid A and ulvanobiuronic through β–elimination reaction | 67 |
| Glucuronan lyase | Filamentous fungi | Acetylated and deacetylated glucuronans in general | 68 |

## 7. Properties

The particular composition and conformation of a given polysaccharide constitute the basis of its physicochemical and biological properties, as well as its particular function on the organism.[72-74] These unique properties, which distinguish each polysaccharide, define the boundaries of their application development. Within this section some of the most studied properties of ulvan are reviewed, with special focus to its molecular weight distribution, ion bonding, ability to gel and cross-link in the presence of ions, as well as an overview of its biological properties.

### *7.1. Molecular weight*

As molecular weight of a polysaccharide is strongly influenced by various factors, including extraction methodology[26, 35, 43], seasonality[34] or algae species and ecophysiology[34], different molecular weights have been reported for ulvan polysaccharide. These may vary from $1.5x10^5$ to $2x10^6$ Da.[31, 35, 44, 75] On the other hand,

ulvan exhibits an aggregation tendency, which can affect molecular weight determinations.[75] Presence of contaminants, different molecular weight distributions or the occurrence of varying ulvan species with variable sugar content and distribution can also influence this property and may explain the polymolecular character of ulvan.[37]

Nevertheless, it is agreed that ulvan is composed of two major macromolecular populations, identified as a high molecular weight fraction ($5x10^5$ to $8\ x10^5$ Da) and a medium molecular weight fraction ($1.5x10^5$ to $2x10^5$ Da), being the high molecular weight fraction the most abundant and the one with higher viscosity.[43, 55, 76]

### *7.2. Selective ion binding*

Green algae are well known for their ability to bind heavy metals, through covalent, electrostatic or redox reactions, removing them from contaminated waters.[5, 77-78] This ability is reported to be associated with the anionic polysaccharide ulvan, present in the cellular walls of green algae, which is rich in functional groups with oxygen, including sulphate or hydroxyl groups, as potential metal binding sites.[5, 77-80] In this regard, ulvan demonstrates different affinities towards various ions, such as Al > Cu > Pb > Zn > Cd=Mn > Sr > Mg=Ca.[37] In the particular case of copper (II) ion, both ulvan's uronic acids and sulphate groups participate on the fixation of this ion.[31, 75]

Besides the environmental impact related with this affinity to complex different ions[81], this property can be directed and applied in a biomedical context. In this context, ulvan can be used as a chelating agent in therapies where the removal of these ions from the body is required or to warranty the presence of ions when they are needed. In this regard and due to this particular property, marine origin polysaccharides, like carrageenan[82] and chitin or chitosan[83], are often proposed as compounds with medicinal or pharmacological interest.

### *7.3. Gel formation and ionic cross-linking*

An important characteristic to define future applications of a polysaccharide is its gelation mechanism. The use of ulvan as a gelling agent would increase its applicability range and encourage the exploitation of green algae.

Ulvan is soluble in water and its solubility may be enhanced with temperature.[84] However, it cannot markedly thicken aqueous solutions and forms weak gels in deionized water, susceptible to pH and ions, with low intrinsic viscosity.[9, 76, 85]

When in aqueous solution, ulvan tends to arrange in a bead-like structure, partially linked by filaments.[76] This peculiar behaviour is explained by a localized hydrophobic

character present within this charged polysaccharide, mostly related with the presence of hydrophobic methyl groups of rhamnose.[76] In this context, water can be considered as a poor solvent for this polysaccharide and may explain the low viscosities observed for ulvan aqueous solutions.[26, 76] Decreasing the pH towards acidic moieties, forces ulvan beads to disperse into an isolated form.[76] Increasing solution pH, promotes ionic interactions between carbonyl and sulphate groups, resulting in aggregation of ulvan beads.[76] The same behaviour is observed in ulvan saline solutions or in the presence of boron or copper, where polysaccharide-self associations are enhanced.[31, 75-76, 86]

In general, ulvan gels are formed in the presence of boric acid and divalent cations, or copper, at alkaline pH.[9, 30-31, 37, 76, 86-87] An important research work of Lahaye and Axelos[9] determined the gelation kinetis of ulvan in the presence of both boron and calcium ions, which lead to the conclusion that gelation of this polysaccharide is time and pH dependent. In an appropriate ionic milieu, this polysaccharide forms a gel and the reaction mechanism is thought to involve the cross-linking between ulvan and boron, mediated by calcium ions.[9, 30, 87] These systems behave as viscoelastic solids, this is to say that they are true gels. A mechanism of gelation of ulvan in the presence of boron and calcium was proposed to require the presence of free *cis*-hydroxyls, involving rhamnose or uronic acids.[9] However, continuous research on this matter revealed that this scenario does not truly represent the gelation reaction mechanism. High sulphation degree of ulvan, mostly localized in rhamnose, may difficult cross-linking which suggests that this particular sugar residue may not be involved in the gel formation through boron interactions.[30] On the other hand, no borate complex was detected, neither involving glucuronic acid nor iduronic acid.[30] In this scenario, the particular gelation mechanism of ulvan in the presence of boron is yet to be unveiled and may involve minor sugars also present within ulvan's backbone.[30]

In agreement with the involvement of ulvan in the ability of green algae to bind metal ions, Paradossi and co-workers found an affinity of this polysaccharide towards copper (II) and Lahaye and co-workes were able to induced gelation of ulvan in the presence of copper, zinc, magnesium and calcium.[31, 37]

The peculiar gelation behaviour of ulvan may result from the aggregation of ulvan bead-like structures through hydrophilic moieties present within this polysaccharide.[76] Nevertheless, viscosity of polysaccharide solutions and gel forming ability are highly dependent on the chemical composition of the polysaccharide itself.[34] In the particular case of ulvan, high uronic acid content seems to affect the viscosity of ulvan solutions and the ability to form gel.[44] In fact, gel formation depends on intra- and inter-molecular cross-links, which are hampered by highly negative groups, including carboxylic acids

(like uronic acids), sulphate groups and/or methyl groups (in rhamnose).[86] An example of the striking effect of sulphation degree on the gelation ability of a polysaccharide is carrageenan. The three main types of carrageenan are kappa, iota and lambda and the main difference between these polysaccharides is their increasing sulphate content.[88] Both kappa and iota carrageenan adopt an ordered double helical structure and form thermoreversible gels through coil-helix conformational transition.[88] However, the higher sulphation degree of lambda carrageenan impedes helix formation and consequently gel formation, possibly through steric hindrance or electrostatic repulsion.[88] Despite the results and hypothesis gathered concerning ulvan's gelation, this is still an open field of research and the mechanisms of gelation are yet to be fully understood.

### *7.4. Biological properties*

Marine algae constitute a rich and largely available source of sulphated polysaccharides with peculiar structures associated with different biological activities.[56, 74, 89] These biological activities have been the focus of intense research and different structure-function studies reveal that these are many times correlated with the sulphation degree of these polysaccharides.[74, 89]

In general, polysaccharides are regarded as non cytotoxic nature origin polymers.[32] Ulvan in particular was already studied and its toxicological effects revealed.[90] It was characterized in terms of its biological performance, evaluated by means of *in vitro* cytotoxicity assays, and demonstrated that this polysaccharide is cytocompatible and is considered non-toxic in the range of concentrations studied.[90] Furthermore, its cellular effect was similar to hyaluronic acid, used as control on their studies.[90] However, a change in the toxicity of this polysaccharide towards colonic epithelial cells was detected for modified ulvans, with low content or reduced uronic acids or desulphated ulvans.[59]

Ulvan has been described as a heparinoid agent, this is to say that possesses biological activity similar to heparin being a potent anticoagulant.[60, 91-92] It also has been found that this polysaccharide is a potent anti-viral, particularly against influenza virus, both human and avian, and herpes simplex virus 1.[59-60] Furthermore, it presents antihyperlipidemic properties, and both ulvan and its low molecular weight oligosaccharides demonstrate an effect on lipid metabolism, limiting hyperlipidemy.[5, 93-95] Its antiperoxidative and antihyperlipidemic properties have shown to exert a protective effect over the liver of rats exposed to a hepatitis-inducing toxin.[33, 95] Antioxidant activity was additionally recognized in ulvan, namely scavenging activity

towards superoxide and hydroxyl radicals, metal chelating activity and reducing power.[60, 96-100] This trait is influenced by the molecular weight of the polysaccharide and its oligosaccharides as well as by the sulphate content of ulvan and its derivatives.[96-97, 100] Chemical modifications of the natural polysaccharide, such as acetylation and benzoylation described by Qi and co-authors[100-101] may enhance this ability. Another important feature of ulvan is its immunostimulating ability, comparable to other algal polysaccharides.[69, 102-103] It has been reported to act as an inflammatory mediator, inducing respiratory burst in flatfish and mammalian phagocytes.[69, 102-104] Phagocyte activation is a key process in the host defence against microorganisms and it is mostly related with the antioxidant properties already recognized in this polysaccharide.[69, 102-103, 105] This activity is strongly influenced by the presence of sulphate groups as well as by exposure time and concentration and may occur through interaction of the polysaccharide with cellular surface receptors.[69, 102-103, 105] Ulvan can also mediate intestinal epithelial growth and take part on the repair of wounds by stabilizing and promoting binding of relevant growth factors to intestinal cells.[84] It has demonstrated antiproliferative activities against human cancer cells, in a dose-dependent manner, particularly for breast adenocarcinoma cells.[60] This may be correlated with its sulphate content and richness in uronic acids.[60]

As mentioned before, many of ulvan's biological properties are strongly influenced by its sulphation degree. However, these can also be related to the ubiquitous presence of rhamnose within the backbone of this algal polysaccharide.[106] In general, rhamnose-rich polysaccharides possess anti-inflammatory properties, reduce bacterial adhesion to the skin, protect it from UV-induced and age-related damage and stimulate cell proliferation and collagen biosynthesis.[106-107] In fact, skin keratinocytes and fibroblasts possess lectins that recognize rhamnose moieties present within a polysaccharide.[106-107] These peculiarities involve this type of polysaccharides with a unique interest for the treatment of skin pathologies, particularly the ones related with age and its effects.[106-107]

Marine origin polysaccharides are many times associated with important biological activities, which are affected by different factors, including molecular weight, chemical composition and chain conformation.[56] Ulvan is no exception and, as it happens with many other polysaccharides, these properties justify the applicational interest on this green algal polysaccharide.

## 8. Applications

The interest in marine algae, as sources of unique polysaccharides with novel structures and interesting biological activities for innovative potential applications, is increasing. These include food, pharmaceutical and medical industries as well as microbiology and biotechnology applications.[5] However, few of the world's available algae species are used commercially. Among the three main divisions of macroalgae (Chlorophyta, Phaeophyta and Rhodophyta), green algae remain largely unexploited in this area. Gosselin and co-workers[17] noticed, in the early 1960's, the discrepancy in knowledge of green algae's polysaccharides when compared to brown and red algae. Almost half a century later, this is still a quite valid consideration. Although the knowledge has evolved, green algae are still a rather underexploited biomass. Its polysaccharides remain in the field of possibilities, against, for instance alginate and carrageenan or agar. These are well established and worldwide accepted polysaccharides for diverse applications, ranging from food industry to biomedical field.[5, 7, 108-109]

Ulvan from different sources is demonstrating ubiquitous potential to be successfully used in various applications. In this section, the applicability of ulvan as a polysaccharide will be discussed, although one should bear in mind that ulvan oligosaccharides also demonstrate interesting properties that may justify their research and future application development, particularly those related with their biological properties.[93, 97, 110-111]

### *8.1. Active agent for pharmaceutical applications*

A review of algae applicability focused on pharmaceutical and medical applications was written by Albertus Smit, who enhances the ecological significance and potential of algae: "Phycologists may be surprised to discover how frequently seaweed natural products are discussed in medicine".[112]

In ancient times, green algae were used to treat different pathologies, including hyperlipidemia and urinary diseases.[93] Nowadays the medicinal interest in green algae is centred in its polysaccharidic part, particularly ulvan, to be used as a therapeutic active agent.

The presence of glucuronic and iduronic acids makes ulvan a very special polysaccharide. This fact gains importance as we think of this polysaccharide for pharmaceutical and biomedical applications. Both these sugar residues are important constituents of mammalian glycosaminoglycans, including heparin and chondroitin

sulphates.[19, 53, 69] Another remarkable property of ulvan is its sulphation degree. Sulphate groups have long been associated with different biological activities.[74] In fact, sulphated polysaccharides are abundant within animal cells and participate in cell recognition, adhesion or regulation of receptor functions.[69, 94]

The use of ulvan as a strategic alternative to various synthetic or animal bioactive agents would take advantage of its algal origin, together with high availability and low expected production costs, low cytotoxicity and broad spectrum of biological activities. It could be applied as an antiviral[59-60], antioxidant[96-101], as an anticoagulant in alternative to heparin[60, 91-92], antihyperlipidemic[5, 33, 93-95] or make use of its antiproliferative activity towards cancer cells[60] or on the therapy of diseases where the immune system is impaired[69, 102-103]. Furthermore, due to its similarity with mammalian glycosaminoglycans, it could be exploited as a pharmaceutical where the delivery of glycosaminoglycans is needed, such as for the treatment of musculoskeletal disorders.[113-114] On the other hand, rhamnose moieties ubiquitous in ulvan's backbone, as mentioned above, may be the basis for its use for the treatment of skin pathologies, particularly the ones related with age and its effects.[106-107] Massarelli and co-workers[115] have studied the interaction of ulvan with hepatocyte lectins and found that the presence of xylose within the backbone of this polysaccharide mediates the interaction with these membrane receptors. As ulvan is readily recognized by hepatocyte membrane receptors, it could be used as a biomaterial for diagnostic or therapeutic purposes.[115] Moreover, and taking advantage of ulvan's ability to complex with metal ions[37], it can find applications where the removal of these ions from the body is required or to warranty the presence of ions when they are needed. For example, it can be used in the therapy of metal poisoning or even be used as part of drugs relevant for targeted radioactive treatment of tumours, as already proposed for carrageenan.[82] Ulvan's biological activities and possible applicative scenario are summarized in Table 1.3.

**Table 1. 3.** Summary of relevant biological activities associated with ulvan and its potential strategic application in a pharmaceutical context, according to diverse studies reported in the literature ([5, 33, 59-60, 69, 91-103, 116-119]).

| Biological Activity | Strategic Possible Applications | References |
|---|---|---|
| Antiviral | Treatment of viral infections, particularly influenza and HSV-1 | 59-60, 119 |
| Antioxidant | Prevention of oxidative stress and be used as a protective drug for several pathologies, including age related or cancer | 96-101, 116-117 |
| Anticoagulant | Surrogate of heparin | 60, 91-92, 116-118 |
| Antihyperlipidemic | Regulation of lipid metabolism | 5, 33, 93-95, 116-117 |
| Immunostimulating | Therapy of diseases where the immune system is impaired | 69, 102-103, 117 |
| Antiproliferative towards cancer cells | Agent for inhibition of cancer cells proliferation | 60 |

### *8.2. Medical devices*

Side by side with its biological properties and potential pharmaceutical relevance, one can think of ulvan for biomedical applications, in particular for the production of medical devices. In this regard, the technological development of ulvan is still in the field of possibilities and mainly focused on its applicability as a biomaterial for tissue engineering and regenerative medicine. Within a tissue engineering and regenerative medicine context the main objective is to guide cells into forming a functional living tissue.[71] A common strategy involves the use of biodegradable scaffolds that provide structure and support cell adhesion, differentiation and proliferation.[71] Depending on the target tissue one is trying to repair, regenerate or substitute, polysaccharide based systems can assume different forms, with different functionalities, ranging from nano-particulate structures to complex 3D scaffolds, passing through smart systems. Natural origin materials present the additional advantage of possessing a variety of distinctive biochemical cues that may enhance and define their applicability in a biomedical context.[71, 120-121] The promise that tissue engineering and regenerative medicine holds is becoming more realistic as significant milestones are achieved, particularly by the approval and commercialization of different developed systems based on natural polymers. Examples of commercial medical devices include CollaGuide™ and Novocart 3D Autologous Chondrocyte Transplantation, based on collagen, which are used for guided tissue regeneration and cartilage engineering, respectively, and alginate and chitosan wound dressings (Tegagen™ Alginate Dressing, Sorbsan® and ChitoFlex®).

In this area, as it happens in a general applicative overview, when one thinks about marine origin polysaccharides, both research and market are generally based on chitin and chitosan[122-126], carrageenan[123, 127-129] and alginate[129-130, 71, 121, 131-132].

The use of ulvan as a medical device, for applications such as tissue engineering and regenerative medicine, is at its early stage, particularly on what concerns polysaccharide modification and processing and biomaterial design. Nevertheless, diverse ulvan structures for different end applications have been so far developed and are already reported in the literature. These include nanofibres[87], membranes[133], particles[134], hydrogels[135] and 3D porous structures[136]. In order to produce these structures, ulvan has been functionalized by grafting with methacryloyl groups[135] or by cross-linking with butanediol diglycidyl ether[133, 136] and processed into hydrogels by photopolymerization[135], membranes by solvent casting[133], 3D porous structures by freeze drying[136], particles by electrostatic interaction with a polycation[134] and nanofibres by electrospinning[87]. An overview of these structures is presented in Table 1.4. Proposed applications include drug delivery, wound dressing or bone tissue engineering.[87, 133-136]

**Table 1. 4.** Structures based on ulvan developed for biomedical applications, including tissue engineering and regenerative medicine, as reported in [87, 133-136].

| Structure | Processing Methodology | References |
|---|---|---|
| Nanofibres | Electrospinning of a blend solution based on ulvan and poly(vinyl alcohol) | 87 |
| Membranes | Modification of ulvan by chemical cross-linking with butanediol diglycidyl ether followed by solvent casting | 133 |
| Particles | Extrusion-dripping method to form ulvan particles by electrostatic interaction with a polycation (chitosan) | 134 |
| Hydrogels | Modification of ulvan with methacryloyl groups followed by photopolymerization | 135 |
| 3D porous structures | Freeze-drying of a solution prepared with ulvan cross-linked with butanediol diglycidyl ether | 136 |

### *8.3. Personal care products*

The array of personal care products is quite broad and there is a tendency towards the use of natural origin products, including algae extracts.[4, 137-138] The use of ulvan in this particular industry is poorly described, being mostly limited to patents that illustrate and claim the use of ulvan, or a water extract from green algae, in this field.[139-141] However, it is easy to envision the potential applicability of this polysaccharide in personal care products, especially if one considers its described biological properties. First of all, ulvan presents similarities with glycosaminoglycans, like chondroitin and dermatan

sulphate, ubiquitous in skin tissue.[19, 53, 69, 142] Furthermore, being a polysaccharide rich in rhamnose moieties, it may induce cell proliferation and collagen biosynthesis.[106-107] On the other hand, has proven antioxidant properties and high hydration ability.[5, 96-101] In fact, the presence of glucuronic acid within ulvan chain confers moisturizing properties to this polysaccharide, important in protecting skin and preventing damage by exposure to dry environments.[138] These particularities reinforce ulvan's value for cosmetic applications.

### *8.4. Food industry*

Green algae are present in the diet of humans since ancient times, as they are considered as food and an alternative and rich source of vegetables.[5, 7, 21, 62] They are traditionally consumed in Asia and are also approved for consumption in Europe, namely in France.[5, 21, 62, 93, 143] These algae are rich in soluble and insoluble dietary fibres and important minerals, as well as high potencies of vitamins, polysaccharides, chlorophyll and protein, and low lipid content.[5, 62, 93, 144] In fact, nutritional interest in algae resides on their wealth on vitamins, oligoelements, minerals and dietary fibres.[85, 144] On the other hand, algae are considered as low energy food due to its low lipid content and due to the fact that their carbohydrates are resistant to digestion and fermentation.[5-6, 28, 64, 145] Besides consumption of algae, one can make use of its constituents for food related applications. The majority of algal polysaccharides, including ulvan, are resistant to endogenous human digestive enzymes[3, 6, 20] and for this reason, they can be considered and studied as good sources of dietary fibres. [5-6] These fibres are present in algae in larger quantities than the ones found in land plants and can be divided into water soluble and insoluble fibres.[6, 28, 62, 85] Ulvan is recognized as a soluble dietary fibre[6, 28, 64] and it is resistant to hydrolysis by digestive enzymes. Its digestive fate has been proposed to be mostly related with depolimerization and fermentation by the large intestinal bacteria.[3] However, ulvan demonstrates low fermentability and is poorly degraded by faecal bacteria.[28, 64] This trait is strongly influenced by its characteristic chemical structure, with little influence of sulphate groups on the resistance of this polysaccharide to bacterial degradation.[64] In fact, its resistance to degradation is maintained after the removal of sulphate groups or oxidation of uronic acid and can be associated with the absence of ulvan's specific depolymerases in colonic microflora.[22, 146] This data is in accordance with the fact that different bacteria present in human flora do not use ulvan as a source of carbohydrates.[147] Even though ulvan is poorly fermented, it induces positive effects on the metabolism of colonic microflora, including regulation of the activity of the enzymes β-glucuronidase and β-glucosidase[28] and induction of intestinal mucin secretion[148].

Interestingly, it appears that sulphate groups are not the main responsible for some of these effects.[22, 64, 148] On the other hand, it is important to stress that consumption of *Ulva* sp. and its sulphated polysaccharide ulvan poses no threat to human health, contrasting with other types of algae which may induce toxicological and carcinogenic effects.[28]

Dietary fibres possess many interesting properties which render them well established additives for food industry applications, as thickeners, stabilizers, emulsifiers or as bulking or gelling agents.[5, 20] In general, algae dietary fibres, including ulvan, have remarkable hydration ability, form solutions with different viscosities, are able to interact with relevant biological molecules, including cholesterol, and provide bulk to faeces.[5] Furthermore, ulvan can be used in this particular industrial niche for its antioxidant properties, preventing food deterioration.[89]

### *8.5. Other applications*

Besides pharmaceutical, biomedical or food related applications, ulvan has been studied and can be applied in many other areas, ranging from agriculture to more technical usages.

Plant pathogens pose an important threat to agricultural production. In this regard, elicitors of plant defence mechanisms represent a powerful alternative to pesticides.[149] A good elicitor triggers defence responses and ensures protection to different diseases without disturbing the organism's primary metabolism.[150]

Ulvan has demonstrated to activate signalling pathways of intracellular plant defence and exerted a protective effect against plant diseases, proving its potential applicability as an environmental friendly plant pathogens' control.[150-155] In fact, pre-treatment of plants with ulvan induces plant resistance and reduces the impact and severity of fungal diseases; however and despite this effect on the plant itself, ulvan does not impede mycelia growth.[151-152, 154] This activity may be associated with its sulphate content as well as with the presence of rhamnose and uronic acid in its composition, acting through the jasmonic acid pathway.[153] In a more technical application of this polysaccharide, Castro and co-workers have used ulvan to purify a neutrophil fish mieloperoxidase.[156] This particular application of ulvan is based on its anionic nature and on the fact that this polysaccharide resembles some glycosaminoglycans, enabling the binding of the enzyme via electrostatic interactions and its isolation and purification by affinity chromatography.[156] Furthermore, being largely composed of rhamnose, ulvan can considered a good and cheap raw material to obtain this valuable monosaccharide.[157]

### *8.6. Relevant patents*

The increasing demand for novel polymers side by side with the sustainable exploitation of natural resources is forcing the focus of research towards natural origin polymers, particularly from marine origin. This mining of the ocean's resources is the basis of different emergent industries based on the unique properties of marine origin molecules. Examples of successful enterprises include Porifarma B. V. (The Netherlands), BioTechMarine (France) and Seanergy (Faroe Islands), among others. In the particular case of ulvan, the interest in this polysaccharide is being revealed by the increasing number of patents focused on the different possibilities of application development based on this polysaccharide, ranging from cosmetics, food, agricultural, pharmaceutical to more technical applications. A resume of this intellectual property is disclosed in Table 1.5.

**Table 1. 5.** Summary of intellectual property related with ulvan or its derivatives, according to the following references: [116-119, 139-141, 155, 157-160].

| Product based on/including | Application | Priority | Patent Number |
|---|---|---|---|
| Water extract from green algae | Source of L-rhamnose | 1986 | US4758283[157] |
| Water extract from green algae | Active agent for antiviral drugs | 1987 | EP0295956[119] |
| Water extract from green algae | General use for cosmetic, pharmaceutical, food or agricultural applications | 1989 | WO1991/07946[140] |
| Water extract from green algae | Agent with anticoagulant activity | 1990 | EP0475383[118] |
| Water extract from green algae | Therapeutic agent for treatment of cardiovascular pathologies | 2004 | US2007/0036821[117] |
| Ulvan | Elicitor of plant defence mechanisms for agricultural applications | 2004 | US2007/0232494[155] |
| Ulvan | Use of ulvan as interspacing component to prepare an interspersed clay for cosmetic, pharmaceutical, food or packaging applications | 2004 | WO2006/030075[141] |
| Water extract from green algae | Pharmacological agent for therapy of neuropathies associated with diabetes and preservation of renal function and vasculopathies | 2004 | WO2004/103280[116] |
| Ulvan | Modification of ulvan and use of the resulting product as a surfactant | 2005 | US2009/0299053[158] |
| Ulvan | Cosmetic composition – making-up or caring – for skin and lips | 2005 | WO2007/007294[139] |
| Ulvan | Cleansing and detoxifying agent composed of alginate and ulvan | 2007 | WO2009/027955[159] |
| Water extract from green algae | Agent for topical application for the treatment of hot flashes | 2008 | WO2009/142745[160] |

## 9. Outlook and perspectives

The interest on marine origin molecules is not recent and has always attracted attention of visionary entrepreneurs for innovative industrial applications. Within this context, and given the evolution and advances of marine origin polysaccharides of different sources, it is surprising to see the limited evolution of green algae polysaccharides. This is particularly striking if one considers that the study of green algae polysaccharides is being documented since the beginning of the XX century. A possible justification for this impaired development may reside on the peculiar and complex structure of ulvan, only completely unravelled in 1997 with the definite confirmation of the presence of iduronic acid within ulvan's backbone.[19] It was only in 2009 that Robic and co-workers shed some light on the abnormal behaviour of this polysaccharide in aqueous solutions.[76] This knowledge has strong impact on the future application development of this polysaccharide. Since the early 90's, research focused on ulvan has been increasing, with a particular focus on its structure and composition, as well as on its properties. Interest on the application development is far more recent. However, the potential of this green algae polysaccharide is such that intellectual property is being reported since mid 80's. When compared with other polysaccharides originated from red and brown algae, ulvan may be considered as a late bloomer. However, the limits of its potential applicability are being drawn and the knowledge generated during the past few years will drive research a step further towards the application development of this green algae polysaccharide.

## 10. Final Remarks

As the fundamental knowledge on ulvan increases, research on this polysaccharide tends to shift towards applied science. However, this evolution is naturally impaired by a lack of a standardized commercial form of this polysaccharide. In this regard, the design of a novel and effective extraction procedure, focused towards targeted applications, becomes crucial. This certainly will open the way to the possibility of scaling up. This is an important consideration, if continuous research on ulvan is intended.

Literature is rich on reports highlighting the peculiar nature and diverse properties associated to this polysaccharide. In fact, a large portion of research efforts are focused on the study of ulvan's chemistry and properties. This knowledge constitutes the fundamental basis that supports applied studies in an attempt to position ulvan as a valid alternative to other polymers, in diverse areas of knowledge. In this sense, this

polysaccharide can find niches of application in areas like the pharmaceutical industry or the biomedical arena, for instance as a bioactive compound or as a medical device, or find applicability in the personal care products' market, or in the food industry or even in agriculture. However, it's the authors' belief that a practical understanding of ulvan's applicability is still a rather open field of research. This is of course enhanced by the particular demands of each niche of application. For example, if one considers ulvan in a biomedical context, to be used as a medical device for regenerative purposes, considerable research endeavour is still needed to accomplish clinical relevance based on ulvan. In this particular context, research strategies may be focused, for instance, on polysaccharide modification, processing and material design and/or material-cell interactivity in order to achieve successful development.

Although the task of translating fundamental research on ulvan into practical achievements appears substantial, this polysaccharide holds great potential and versatility. Taking advantage of the knowledge gathered research can now be driven towards practical applications of ulvan, in an attempt to decrease the distance between scientific understanding and industrial awareness. The promise of success is feasible and for now, one can only say: "*alea jacta est"*.

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# SECTION II – DETAILED DESCRIPTION OF EXPERIMENTAL TESTING AND MATERIALS

# Chapter 2

# Materials and Methods

## Materials and methods

More than a summarized description of the materials and methods employed throughout the herein presented work, this chapter intends to present in more detail the materials and techniques chosen to accomplish the proposed objectives. This allows for a deeper understanding of experimental and analytical tools utilised.

## 1. Materials

### *1.1. Ulva lactuca*

Ulvan was extracted from the green algae *Ulva lactuca*. Unless otherwise stated, these algae were supplied by Setalg (France) as dry flakes with a moisture content ≤15%. These algae are supplied with a certificate analysis report as follows (Table 2.1):

**Table 2. 1.** Setalg's *Ulva lactuca* certificate analysis.

| **Toxicological Analysis** | |
|---|---|
| **Mineral Arsenic (As)** | In conformity (Standard ≤ 3mg/kg) |
| **Cadmium (Cd)** | In conformity (Standard ≤ 0.5mg/kg) |
| **Mercury (Hg)** | In conformity (Standard ≤ 0.1mg/kg) |
| **Iodin (I)** | In conformity (Standard ≤ 2000mg/kg) |
| **Lead (Pb)** | In conformity (Standard ≤ 5mg/kg) |
| **Tin (Sn)** | In conformity (Standard ≤ 5mg/kg) |
| **Microbiological Analysis (Standards according to Conseil Supérieur d'Hygiène Publique de France)** | |
| **Total microorganisms obtained at 30ºC** | In conformity (Standard ≤ 100000 UFC/g) |
| **Thermotolerant coliforms** | In conformity (Standard ≤ 10 UFC/g) |
| **Anaerobic sulfito-reducing bacteria** | In conformity (Standard ≤ 100 UFC/g) |
| ***Staphylocoques coagulase* +** | In conformity (Standard ≤ 100 UFC/g) |
| ***Clostridium perfringens*** | In conformity (Standard ≤ 1 UFC/g) |
| ***Salmonella*** | In conformity (Standard Abs/25g) |

#### *1.1.1.Ulvan*

Extracted ulvan has been characterized and some of its properties and composition can be summarized as follows (Table 2.2)[1]:

**Table 2. 2.** Properties and composition of ulvan extracted from the green algae *Ulva lactuca*.[1]

| **Ulvan extracted from *Ulva lactuca*** | |
|---|---|
| **Molecular weight** | 790 kDa |
| **Protein** | 1.3% |
| **Sulphate** | 32.2% |
| **Sulphate free inorganic material** | 10.3% |
| **Rhamnose** | 22.4% |
| **Glucuronic Acid** | 22.5% |
| **Xylose** | 3.7% |
| **Iduronic Acid** | 3.1% |
| **Glucose** | 1% |

### *1.2. Hyaluronic acid (HA)*

Hyaluronic acid is a linear polysaccharide, member of a class of polymers known as glycosaminoglycans (GAG's). This polysaccharide is composed of β-(1→3) linked N-acetyl-D-glucosamine and β-(1→4) linked D-glucuronic acid residues (Figure 2.1) and can be isolated from various animal tissues or obtained by bacterial fermentation.[2]

This is a polysaccharide with ubiquitous usage in the biomedical and non-medical field, due to its remarkable physicochemical properties reinforced by its non-immunogenic nature.[2-4] It is considered as a gold standard for many applications and for this reason it was chosen as a non-cytotoxic control in the study of ulvan's cytotoxicity (Chapter 4).

Hyaluronic acid used in these studies was supplied by Sigma–Aldrich (Germany), as a white powder in the sodium salt form with 1% protein impurities and used without further modifications.

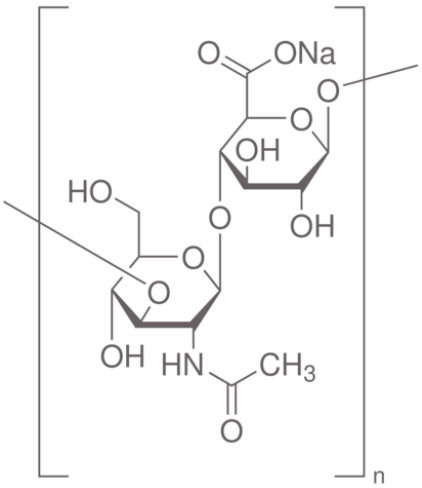


**Figure 2. 1.** Hyaluronic acid disaccharide unit composed of *N*-acetyl-*D*-glucosamine and glucuronic acid.

### *1.3. 1,4- Butanediol diglycidyl ether (BDDE)*

Different cross-linking agents may be used to cross-link ulvan, including 1,4- butanediol diglycidyl ether. This is a homobifunctional epoxide reagent considered cytocompatible and is frequently employed in biomedical, pharmaceutical or cosmetic applications, where it is well established and widely accepted.[5-9]

Both hydroxyl and carboxyl groups present within ulvan's backbone may be utilized for the cross-linking of this polysaccharide. Hydroxyl groups may be cross-linked via an ether linkage and carboxyl groups via an ester linkage. In acidic conditions labile ester bonds are formed, in alkaline media stable ether bonds are obtained and at neutral pH, a mixture of both is obtained.[7, 9-11]

BDDE was supplied by Sigma–Aldrich (Germany) and used without further modifications, with a molecular weight of 202.25 g/mol and a purity grade ≥ 95%. The molecular structure of this reagent is presented in Figure 2.2.

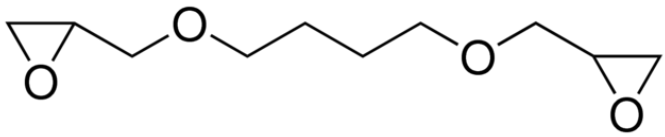

**Figure 2. 2.** 1,4- Butanediol diglycidyl ether structure.

### *1.4. Poly-(D, L-lactic) acid (PDLLA)*

PDLLA is an amorphous derivative of poly-lactic acid, easily obtained from bacterial fermentation.[12-14] This polyester is hydrophobic, biocompatible and bioresorbable, being believed to degrade in the body into non-toxic lactic acid residues.[12, 14-15] It is this known degradability associated with various physical, thermal, mechanical and biological properties that justifies intense research and use of this polymer in human therapy.[12, 15]

PDLLA was supplied by Purasorb (The Netherlands), in the form of white granules with an inherent viscosity of 1.87dL/g. It was used to produce porous scaffolds by a subcritical sintering technology (Chapter 7). Molecular representation of the repeating unit of PDLLA is depicted in Figure 2.3.

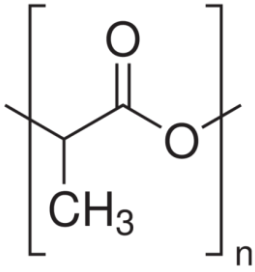


**Figure 2. 3.** Structural unit constituent of Poly-(D, L-lactic) acid.

### *1.5. Chitosan*

Chitosan is the semi-synthetically *N*-deacetylated polysaccharide, derived from chitin, a natural polymer present in different organisms including the exoskeleton of insects, crustaceans and fungi.[16] This is a linear polysaccharide composed of 2-acetamido-2-deoxy-*D*-glucopyranose and 2-amino-2-deoxy-*D*-glucopyranose units linked by β-(1→4) glycosidic bonds (Figure 2.4).[17] The cationic character of chitosan confers good complexing ability to this polysaccharide which can be used on conjugation studies with other polysaccharides, including ulvan.[18]

Chitosan was purchased from Sigma-Aldrich (Germany) with medium molecular weight and purified through a re-precipitation method, described by Signini and Filho.[17] Briefly, a solution of chitosan 1% (w/v) in 2% (v/v) aqueous acetic acid is prepared and filtered twice to remove any insoluble material. Chitosan is precipitated with 1M aqueous sodium hydroxide. Precipitate is recovered by filtration, extensively washed with distilled water, until a neutral pH is reached, and freeze dried. White flakes of this polysaccharide are obtained.

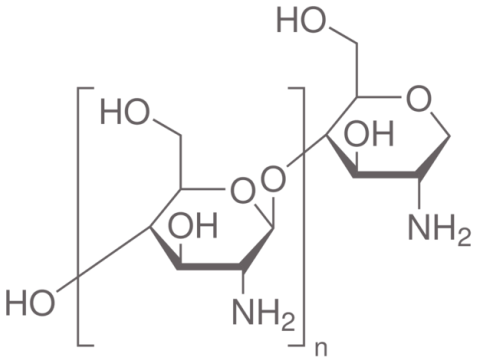


**Figure 2. 4.** Chitosan disaccharide unit composed 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units linked by β-(1→4) glycosidic bonds.

## 2. Extraction of ulvan from green algae

Nowadays, most of materials used for research in a biomedical context are commercially available. However, ulvan still needs to be extracted from green algae as it is not yet commercialized.

In order to obtain this polysaccharide a novel extraction methodology has been developed. A detailed description of extraction methodologies employed on the extraction of ulvan from green algae was already given in Chapter 1. For this reason, the general procedure herein used to obtain ulvan will be briefly described in this section.

As a natural product extract, the influence of several interfering substances has to be recognized and these have to be removed whenever and as efficiently as possible. In a first stage, dried *Ulva* sp was submitted to Soxhlet extraction with organic solvents in order to remove lipids and colouring matter. The choice of this technique relays on its recognition as one of the most efficient procedures to remove contaminants from a sample.[19-20]

Following Soxhlet algae pre-treatment, polysaccharide *per se* was extracted from the residual off-white dried weed with hot water, in the range of 75-90ºC, based on ulvan's solubility in this particular solvent.[21] These conditions allow the extraction of a high molecular weight polysaccharide with a suitable yield.[22-24] Although several additives may be used to increase the yield of extraction[23, 25-30], the choice of hot water as extraction solvent reduces introduction of contaminants during extraction.

Obtained aqueous extracts are centrifuged, filtered and concentrated in a rotary evaporator. Resulting aqueous extract solution is further purified through enzymatic

hydrolysis with salivary α-amylase and proteinase K in order to remove starch and peptides, respectively, both typical contaminants which may be co-extracted with this polysaccharide. This methodology is frequently used and already proven effective.[1, 22, 31-33] Small molecular weight contaminants, including the ones responsible for odour and also colour can be removed with activated charcoal, through selective adsorption.[20, 27, 31] Finally, resulting solution is subjected to dialysis (salting-out), precipitated with ethanol and freeze dried.[34]

A schematic summary of the overall extraction design is depicted in Figure 2.5.

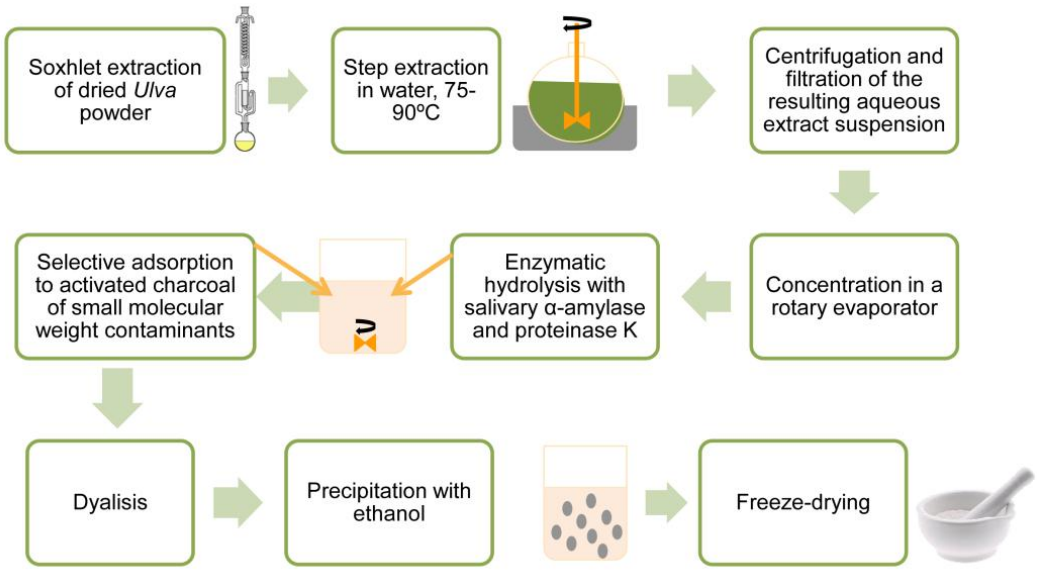


**Figure 2. 5.** Extraction methodology employed to obtain ulvan from the green algae *Ulva*.

## 3. Polysaccharide characterization

It is indeed a challenging endeavour to characterize a natural origin polysaccharide.

After a concise understanding of extracted ulvan's structure and some of its chemical characteristics (work not included in the present thesis)[1], a direct characterization was performed by focussing on particular characteristics regarded as important for the application development of this polysaccharide.

### *3.1. Elemental analysis and protein quantification*

Elemental analysis, as a type of combustion analysis, is widely used for the determination of the percentage of different atoms within the sample. Samples are completely burned and the elemental composition (carbon - C, hydrogen - H, sulfur - S

and nitrogen - N) of the polysaccharide is determined by quantitative measurements of the combustion products, namely $CO_2$, $H_2O$, $SO_2$ and $N_2$. C, H, S and N contents present on ulvan samples were quantified by combustion of the sample, using a Carlo Erba CHNS-O EA 1108 apparatus.

The percent composition of nitrogen present in the analysed ulvan allowed the determination of protein content, through Kjedhal analysis by using a conversion factor to convert the measured nitrogen content to a protein percent composition.[35] This method is widely used as a standard and simple estimation of protein content in foods. However, it does not give a measure of the true protein content in a given sample, as it also accounts for non-protein nitrogen. Nevertheless, according to this method, it is possible to calculate the amount of crude protein (CP) in the sample by multiplying the percent nitrogen (N) by a conversion factor (6.25).

$$CP = N \times 6.25$$

### *3.2. Fourier transform infrared spectroscopy (FTIR)*

FTIR is often used due to the fact that it constitutes a quick and low-cost method. It is based on the vibrational excitation of covalently bonded atoms and groups present in molecules exposed to infrared radiation.[36-37] FTIR spectrum is obtained through the determination of absorbed incident radiation at a given energy and corresponds to the frequencies of vibrations between the bonds of the atoms present within the molecule.[37] In this sense, it represents a molecular fingerprint of the analysed sample.

In order to identify the fundamental and characteristic functional groups present in ulvan's structure, FTIR has been performed. Extracted polysaccharide powder was analyzed using an IR Prestige-21 apparatus (Shimadzu, Japan). Pre-dried powder was mixed with potassium bromide (KBr), in a mixing ratio of 1:10 of sample:KBr (w/w), and formed into a transparent pellet by manual uniaxial pressing. Transmission spectra were recorded using at least 32 scans with 4 $cm^{-1}$ resolution in the spectral range of 4000 – 400 $cm^{-1}$.

### *3.3. Nuclear magnetic resonance (NMR)*

Proton NMR spectroscopy is a fundamental tool when studying the chemistry of polysaccharides as it allows identification and the study of their structure. This technique exploits the properties of magnetic activated atomic nuclei possessing spin.[38] Polysaccharides are rich in $^{1}H$ and $^{13}C$, which are considered as NMR-active nuclei.[39] These activated nuclei absorb electromagnetic radiation at a characteristic frequency and the transitions between energy levels are determined.[38]

At this point it is important to stress that due to the high number of possible disaccharide sequencings and distribution in addition to other possible structural irregularities or contaminants, obtained polysaccharides spectra are many times intricate, which enhances the complexity of analysing the signals.[40-41] Despite the complexity of carbohydrate NMR spectra, information can be obtained about the chemical composition of the extracted polysaccharide.[39-40] In this particular study, this technique was used to identify the presence of characteristic sugar moieties of ulvan.

$^{1}$H-NMR spectrum of pre-dried polysaccharide (10mg/ml) dissolved in a deuterated solvent (deuterium oxide) was recorded on a Varian Unity Plus (Varian, USA) spectrometer, at 25ºC.

### *3.4. Thermogravimetric analysis (TGA)*

Thermogravimetric analysis provides a quantitative measurement of mass change in materials associated with dehydration, decomposition and oxidation of a sample with time and temperature.[42] For TGA, a small amount (15-18 mg) of powder was taken for analysis in a Perkin Elmer TGA7 (Perkin Elmer, USA). The samples were heated from 40 to 720°C at a rate of 10°C min$^{-1}$ under a nitrogen atmosphere.

### *3.5. Differential scanning calorimetry (DSC)*

DSC is another thermal analysis technique relevant in the characterization of the thermal properties and transitions of a polymer.[42] Important and characteristic polymer thermal transitions analysed with DSC include glass transition temperature (Tg) or melting temperature (Tm).

DSC experiments were carried out using TA-Q100 equipment (TA Instruments, USA), under a nitrogen atmosphere, on samples (5-10 mg) packed in aluminium pans. Samples were heated in two stages at a constant heating rate of 20 ºC/min from -20ºC up to 180ºC, then were left at this temperature for a period of 2 min and cooled at -20ºC/min to the initial temperature. At this point a second heating run was conducted. An empty aluminium pan was used as reference.

### *3.6. Dynamic mechanical analysis (DMA)*

Glass transition is one of the most important parameters in the characterization of a polymer. It occurs in amorphous materials or in the amorphous regions of a semi-crystalline polymer and it is characterized by a relaxation of the polymeric chains. Bellow this temperature, the polymer assumes a rigid and brittle behaviour, being

elastic above this temperature. These changes impact their physical properties and processability.

There are several techniques that can be employed for the study of this particular thermal transition, including DSC and DMA. In a typical DSC trace, a change on the heat capacity is detected upon glass transition; in DMA this transition is detected by a significant drop in the loss modulus or by a concurrent peak in tan delta (Tan δ).[42]

In the present thesis, DMA (TRITEC 8000B DMA apparatus, Triton, UK) was used to investigate the in more detail some of the transitions detected in the DSC trace. Stainless steel pockets, provided by the DMA manufacturer, for these specific measurements (length = 15 mm, width = 7.5 mm, thickness = 1 mm) were used to fix the powder material into the DMA loading frame. Such pocket devices are especially useful to analyse powder materials and are frequently applied in pharmaceutics, for the study of active agents.[43] The pockets filled with the sample were subjected to sinusoidal loading under Single Cantilever Bending mode conditions. The load was automatically adjusted in order to achieve an imposed displacement equal to 0.05 mm. The measurements were performed at frequencies of 1, 5, 7.5 and 10 Hz over the temperature range between 20 °C to 140 °C at 2 °C/min for each frequency. Note that the storage modulus that is given by the apparatus is basically resulting from the properties of the metallic component. However, any change in the viscoelastic properties of the sample will be detected in the tan δ trace.

In order to confirm a glass transition temperature, both storage modulus and tan δ were recorded as a function of frequency, as well as temperature, and an Arrhenius equation was applied in order to determine the activation energy of a given thermal transition.

$$lnf = lnA - \left[\frac{E_a}{R \cdot T}\right]$$

where *f* is the applied frequency, A is a constant, R is the universal gas constant and T is the peak maximum temperature of the tan δ peak. The relationship between the observed changes in tan δ with frequency allows the computation of the activation energy.[43]

### *3.7. X-ray diffraction (XRD)*

X-ray diffraction is another technique that can be employed for bulk characterization of polymers, particularly on the three-dimensional arrangement of molecules. XRD patterns were measured using a powder diffractometer Philips PW1710 apparatus (Philips, The Netherlands), with Cu-Kα radiation at 50 mA and 40 kV, operated in step

scan mode (1.542Å wavelength). The diffraction data was acquired at a rate of 0.02º 2θ/s and over a Bragg angle range of 10º<2θ<50º.

### *3.8. Biological assays*

Prior to any application development, every natural origin polysaccharide should undergo a screening stage of cytotoxicity testing. This procedure allows the detection of detrimental effects of the polymer towards cellular functions.

An *in vitro* approach is economically and ethically favoured over animal tests, in compliance with the 3R's Principle (Reduction, Refinement and Replacement). Overall *in vitro* cytotoxicity procedures have proven to be effective in the screening of human chemical toxicity.[44] In standard tests, the sample is incubated in direct contact with a layer of cells, usually from a cell line, for a period of at least 24 hours, together with positive and negative controls.[45] This is considered the closest assay to evaluate and predict the cytotoxicity and the behaviour of a material in the body, since there is no barrier between the material in study and the cell layer.[44] The effect of the presence of a foreign agent on cellular homeostasis may be detected by the disturbance of normal cellular biochemical functions; this knowledge is the foundation of many standard toxicologic studies (Figure 2.6).[46-47] Cytotoxicity of extracted ulvan was evaluated according to ISO/EN 10993 part 5 guidelines for an exposure period of 24h.[45]

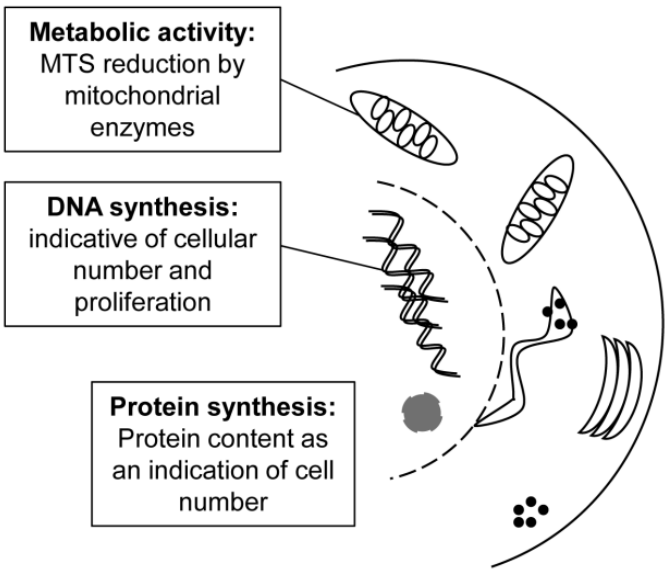


**Figure 2. 6.** Animal cell basic scheme with the indication of some of the targets (biochemical parameters) usually employed on the study of cytotoxicity.

### *3.8.1. Cell lines*

For the present characterization work cell lines were used, namely mouse C3H/An connective tissue fibroblast-like cells (L929), obtained from the European Collection of Cell Cultures (ECCC, UK). The use of continuous cell lines possesses several advantages, such as their abundance, standardization of culture conditions and easy storage.[48-49]

### *3.8.2. Cell culture*

Cells were cultured in tissue culture flasks at sub-confluent cell density in Dulbecco's modified eagle medium (DMEM) supplemented with sodium bicarbonate (44 mM) (Sigma–Aldrich, Germany), 10% fetal bovine serum (FBS – Alfagene, USA) and 1% of antibiotic-antimycotic solution (Gibco, UK). Cultures were maintained at 37°C, in a humidified tissue culture incubator with a 5 % CO2 atmosphere. Medium was renewed every 2-3 days.

### *3.8.3. Polymer solutions preparation*

Cytotoxicity assays were performed using latex rubber and standard culture medium as positive and negative controls, respectively. Latex rubber is commonly used as a positive control for standard toxicological assays, as it is known to have a cytotoxic effect inducing cell death and lysis.[50] Hyaluronic acid (HA), supplied by Sigma–Aldrich (Germany), was additionally used as a non-cytotoxic reference material.

Ulvan and hyaluronic acid were dissolved in DMEM supplemented with sodium bicarbonate (44 mM), 10% FBS and 1% of antibiotic-antimycotic solution. Fresh stock solutions of ulvan and hyaluronic acid were prepared and diluted to the final test concentrations, in order to obtain polymer solutions with concentrations between 0 and 15 mg/ml.

### *3.8.4. Biochemical parameters applied in the study of the cytotoxicity of extracted polysaccharide*

Cell growth in normal culture conditions can be impaired by the presence of foreign compounds if one or more essential cellular functions, such as mitochondrial activity, DNA and protein synthesis or maintenance of membrane integrity, are affected.[51] In fact, loss of viability, reduced proliferation rate or cell membrane rupture represent critical consequences that can result from exposure to a toxic biomaterial.[52] Based on this, several methods can be used to evaluate cell viability and proliferation, like reduction of MTS and quantification of total protein or measurement of DNA synthesis

following the incorporation of radio-labelled molecules.[52] Performed assays are based on metabolic activity given by mitochondrial activity through MTS assay, determination of nucleic acids through dsDNA quantification, and protein content through total protein quantification.

- MTS assay

MTS is commonly used for the analysis of cell metabolic activity and provides an indication of cell viability. This particular assay determines if cells exposed to the different polymers are metabolically active. It is based on reduction of the substrate, 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2(4-sulfofenyl)-2H-tetrazolium (MTS) in a mitochondria-dependent reaction to yield a brown formazan product. This conversion is accomplished via NADPH or NADH produced by mitochondrial dehydrogenase enzymes in metabolically active cells. In this sense, only viable cells, undamaged by a foreign agent, can express this enzymatic activity.[49]

In order to perform this assay, a CellTiter 96® AQueous One Solution Reagent (Promega, USA) was used. Briefly, cells were seeded onto 96-well plates at a concentration of $5x10^3$ cells per well. After 24 h, medium and unattached cells were removed and polymer solutions were added. Culture medium without any polysaccharide was used as negative control and it is represented as 0 mg/ml. Cells were incubated in the presence of the different samples for an additional 24 h period. Three replicates were considered for each sample. After each time of exposure, medium was discarded from each well and the cell monolayer was washed twice with sterile phosphate buffered saline (PBS) solution. Cells were exposed to tetrazolium solution (MTS), following manufacturer's protocol, which allowed for viable cells to reduce the tetrazolium compound into a water soluble formazan product. Optical density at 490 nm was measured with a microplate reader (Synergie HT, Bio-Tek, USA).

- Double stranded DNA (dsDNA) quantification

Double stranded DNA quantification is based on fluorescence enhancement of a dye, PicoGreen, upon binding to dsDNA. The relative fluorescence units measured correlate with the total number of cells.[47] In this sense, measurements of DNA synthesis are frequently taken to be representative and indicative of cell proliferation.[53]

As for the MTS test, after cellular confluence culture medium was replaced by polysaccharide solutions. Three replicates were considered per sample. Culture

medium without any polysaccharide was used as negative control (represented as 0 mg/ml). After 24h of incubation media was discarded from each well and the cell monolayer was washed twice with sterile PBS solution. Cell proliferation was measured by the total amount of double-stranded DNA and quantification was performed using the PicoGreen dsDNA Assay Kit (Invitrogen$^{TM}$, Molecular Probes$^{TM}$, USA), according to the manufacturer's instructions. In brief, cell monolayer was lysed by osmotic and thermal shock and the supernatant used for DNA quantification. Fuorescence was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, in a microplate reader (Synergie HT, Bio-Tek, USA). DNA concentration for each sample was calculated using a standard curve of lambda DNA relating DNA concentration (ranging from 0 to 1.5 µg ml$^{-1}$) and fluorescence intensity.

- Total protein quantification

Total protein, measured through bicinchoninic acid (BCA) assay, combines the protein-induced biuret reaction with the highly sensitive and selective colorimetric detection of the resulting cuprous cation ($Cu^{1+}$) by BCA. The measured protein content corresponds directly to the cell number.[47]

As for dsDNA quantification, after reaching confluence the culture medium was replaced by the polysaccharide solutions. Three replicates were considered per sample. Culture medium without any polysaccharide was used as negative control (0 mg/ml). After 24h of incubation media was discarded from each well and the cell monolayer was washed twice with sterile PBS solution. From this point on, Micro BCATM Protein Assay Kit (Pierce Chemical, USA) was used, following manufacturer instructions. In summary, cells were exposed to a working solution containing bicinchoninic acid (BCA) as detection reagent for $Cu^{+1}$, which is formed when $Cu^{+2}$ is reduced by proteins, producing a purple reaction product. Optical density at 562 nm was measured using a microplate reader (Synergie HT, Bio-Tek, USA). A standard curve, using albumin as standard (0 to 200 µg/ml), was created and sample protein values were read off from the standard graph. Plotting the measured absorbance against albumin concentration allowed the calculation of protein concentration.

## 4. Sample processing

In order to understand the limits of processability of ulvan extracted from green algae, different polymer processing methodologies were attempted.

Ulvan is a polysaccharide that is soluble in water and prone to hydrolytic degradation in physiological conditions. This *per se* justifies the need for a modification step in order to render produced ulvan structures insoluble in water and stable at physiological conditions. Cross-linking was the chosen method to modify ulvan in order to produce both ulvan membranes and 3D porous structures. Among the different commercially available cross-linking agents we have chosen to work with 1,4- butanediol diglycidyl ether (BDDE) due to its ubiquitous applicability and acceptability in biomedical, pharmaceutical or cosmetic applications.[5-8] As a practical example, this agent is widely used to cross-link hyaluronic acid to produce commercially available stable fillers for dermatological purposes.[5]

There are several different processing techniques that can be employed in the processing of ulvan. Among these, solvent casting was performed to obtain ulvan membranes, freeze drying to obtain ulvan porous structures and extrusion-dripping method to produce ulvan particles. Each of these techniques will be further discussed.

### *4.1. Cross-linking ulvan prior to membrane preparation*

Ulvan was mixed with BDDE (1:5 molar ratio), in an alkaline media (sodium hydroxide, 40 mM). The cross-linking reaction was allowed to proceed for 180 min, at 50ºC. Cross-linked ulvan powder was exhaustively washed with water and acetone to remove any residual cross-linker. The obtained dried powder was further used to produce cross-linked ulvan membranes.

#### *4.1.1. Ulvan membranes produced by solvent casting*

Polymeric membranes were produced by solvent casting methodology. In this process, a homogeneous polymer solution is placed in an appropriate mould and the solvent is removed by evaporation. Membrane formation through solvent casting is affected by different physicochemical processes, namely diffusion, mass transfer, convection and precipitation.[54] This is a solvent based technique that depends on polymer solubility and solvent volatility. Properties of the polymeric solution, including the polymer effect, solvent effect and a combined effect of both factors, play a decisive role within this process and on the final properties of the obtained membrane.[54-55]

To prepare the polymeric membranes, cross-linked ulvan was dispersed in water (1% w/v) and the solution was homogenised with an UltraTurrax apparatus. Ulvan membranes were prepared by casting ulvan solution on Petri dishes, followed by solvent evaporation, at 50ºC, in a vacuum oven.

In the present study, dexamethasone, a steroid anti-inflammatory drug, was used as a model therapeutic agent. In this case, ulvan membranes impregnated with dexamethasone were prepared as described above, and dexamethasone was dissolved in acetone and added to ulvan solution, with a final concentration of 15% w/w, prior to solvent casting. The final appearance of ulvan membranes, loaded or not with dexamethasone, is a homogeneous and transparent yellowish film.

### *4.2. Development of chemically cross-linked freeze-dried ulvan porous structures*

Freeze drying is often used to produce porous structures. A polymer solution is prepared, frozen and the solvent removed by lyophilisation under high vacuum.[56] Hence, this technique involves two stages, namely freezing the solvent which can be considered as a crystal nucleation process and sublimation of the resulting ice crystals leading to the formation of pores. This particular methodology makes use of ice crystals as porogens which results in a final porous architecture that is strongly influenced by solution properties as well as by polymer molecular arrangements.

Prior to the processing of the structure *per se*, ulvan was cross-linked with BDDE. This chemical cross-linking was performed by simply mixing the polysaccharide with the cross-linking agent in an alkaline medium, to favour the formation of stable ether bonds.[57] Ulvan (referred as UL) and cross-linker (referred as XL) concentrations are reported in Table 2.3.

**Table 2. 3.** Different formulations based on ulvan (UL) and cross-linker (XL) used to prepare various ulvan porous structures.

| [UL] (% w/v) | XL (w/w) |
|---|---|
| 4 | 0.20; 0.27; 0.53 |
| 5 | 0.14; 0.50; 1.00; 1.50; 2.00 |
| 6 | 0.13; 0.18; 0.36; 0.57 |
| 8 | 0.25; 0.50; 0.75; 2.00 |

The reaction was allowed to proceed for 180 minutes, at 50ºC. Afterwards, the solution is dialyzed against distilled water to remove any unreacted cross-linker, placed in an appropriate mould and freeze dried to allow the formation of porous structures.

### *4.3. Ulvan particles produced by extrusion-dripping method*

Extrusion-dripping method is a technique widely applied to produce polymer particles. These structures are prepared by adding a polysaccharide solution, in a dropwise manner, into a coagulation bath. The contact of droplets with the coagulation bath results in the instantaneous formation of gel-like particulate structures (Figure 2.7). Being a polyanion, ulvan is able to form insoluble complexes when in contact with water soluble polycationic molecules.[58]

In order to produce the envisaged ulvan particles, an aqueous ulvan solution (6% w/v) was prepared and placed in a syringe to be added in a drop wise manner to a chitosan solution (1% w/v in 1% v/v acetic acid). This procedure was performed at room temperature and contact time of ulvan particles with chitosan solution was ~2h, as lower contact times resulted in unstable and rapidly dissolving particles. After stabilization, ulvan particles were collected, extensively washed and dried. Incorporation of dexamethasone was achieved by directly adding it to ulvan solution at a concentration of 15% (w/w).[59]

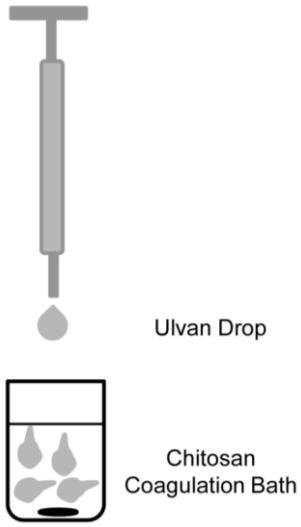


**Figure 2. 7.** Method to produce ulvan particles by addition of an ulvan solution in a drop wise manner to a coagulation bath composed of a polycation.

Produced ulvan particles were afterwards incorporated into a polyester matrix, produced by subcritical sintering.

#### *4.3.1. Polyester matrix produced through subcritical sintering*

Subcritical sintering method uses carbon dioxide in a subcritical state to prepare highly porous and interconnected structures, under mild conditions.[60] This methodology

usually makes use of carbon dioxide which is considered environmentally benign, nontoxic, non-flammable, noncorrosive, readily available, inexpensive and an alternative to the use of organic solvents. The subcritical sintering technique, similarly to the supercritical fluid foaming, relies on the plasticizing effect of carbon dioxide which reduces the glass transition temperature of polymers. In the sintering process, the polymer is plasticized in mild conditions and the polymer particles are fused, creating a 3D scaffold with appropriate morphological properties for tissue engineering and regenerative medicine.[61] This method eliminates the need of organic solvents and high temperatures generally needed to process synthetic polymers, with an additional advantage of allowing the enrichement with labile bioactive agents.[60, 62-66]

Scaffolds were prepared by a subcritical fluid assisted sintering method at 50 bar and 40 ºC. Approximately ~120 mg of a mixture of PDLLA and ulvan particles (empty or loaded with dexamethasone) were positioned in a mould, which was placed in a high pressure chamber. The chamber was heated by means of an electric thin band heater connected to a temperature controller. The sintering occurred within 30 minutes, which was the optimum contact time to allow the plasticization of the polyester; after this, the system was quickly depressurized.

## 5. Sample characterization

Various techniques are available to characterize the produced polymer structures assessing different parameters, including mechanical and thermal properties, morphology, chemical characterization and stability or biological activity. In this context, a strategic approach involves a multi-parameter characterization in order to acquire a realistic overview regarding the material's properties and functionality.

### *5.1. Fourier transform infrared spectroscopy (FTIR)*

The bases of this technique are explained in more detail in section 3.2. Besides polysaccharide characterization, FTIR can also be used to study chemical modifications introduced by, for instance a cross-linking reaction, as part of structure's characterization.

### *5.2. Scanning electron microscopy (SEM)*

Scanning electron microscopy is a valuable method frequently applied to study morphologic properties of polymeric structures.[67] Nonconductive polymeric materials are normally coated with a thin, conductive material, such as gold or carbon by sputtering, in order to minimize negative charge build up from the electron beam.[67]

Developed structures were sputter-coated with gold, prior to SEM analysis (Leica Cambridge, model S360 (Leica Cambridge, England) or FEI Nova 200 (FEI, USA)).

### *5.3. Micro-computed tomography (μ-CT)*

Micro-computed tomography makes use of X-rays to create an image/projection of a 3D object used to construct a virtual model without destroying the original object.[68-69] The sample is defragmented into a series of 2D projections, that are exposed from the edges to X-rays, which are then used to build a 2D pixel map. Further analys with specific software generates a 3D model, representative of the analysed sample.[69] In this context, it is able to provide both quantitative and qualitative information regarding the morphology of the sample. Micro-CT analysis includes information of porosity, mean pore size and pore size distribution, interconnectivity, anisotropy, etc.[69-70] By applying this technique it is possible to scrutinize and visualize the intricate and complex 3D structure of the sample.

Structures herein described were analyzed by micro-computerized tomography using a high-resolution micro-CT SkyScan 1072 scanner (Skyscan, Belgium) to characterize its morphological and morphometric properties. Each scaffold type was scanned in high-resolution mode. Representative data sets of the analyzed sample were converted into binary images using a dynamic threshold to distinguish between polymer material and void space. Those operating parameters were maintained for each set of samples in each particular study. For morphometric analysis (CT Analyser®, SkyScan, Belgium), 2D images were reconstructed using CT Analyzer software from the micro-CT scanner supplier (NRecon, SkyScan, Belgium). Different morphometric parameters were determined, including porosity, mean pore size and respective distribution and structure interconnectivity. 3D virtual models of representative regions in the bulk of the structures was also created, visualized and registered using the image processing software supplied by the manufacture.

### *5.4. Mechanical characterization*

Mechanical properties of different polymeric structures can be characterized by their deformation behavior, resulting in a stress-strain response.[67] These properties are associated with the molecular arrangements of the processed polymer and are crucial in evaluating the effectiveness of the processed structures to be selected for a particular biomedical application.[67]

Mechanical characterization of produced ulvan structures was performed through quasi-static testing in tensile or compression mode, using a universal testing machine.

This technique allows the investigation of the mechanical behaviour of the produced structures under static loading.

### *5.4.1. Tensile testing*

Ulvan membranes described in Chapter 5 were subjected to tensile tests to evaluate the effect of cross-linking over the mechanical properties and to determine the mechanical performance of these membranes. Membranes with 500µm in thickness were cut into 10-mm-long specimens and tensile tests, in dry state, were performed using a Universal Testing Machine (Instron 4505, Instron Int. Ltd., UK), with a load cell of 1 kN, gauge length of 10 mm and cross-head speed of 5mm/min was used up to rupture of the membrane. A minimum of five specimens were tested for each sample (the values reported are the average of those results).

These tests allowed the determination of different mechanical properties, defined as follows and represented in Figure 2.8:

- tangent modulus, defined as the slope of the straight line obtained by linear regression of the stress-strain curve in the near elastic region of the material (between 0 and 1.0 % strain);
- the ultimate tensile strength, defined as the maximum tensile stress developed in the material during the tensile test;
- strain at break point, defined as the maximum strain of the material, i.e. elongation at the failure point of the material.

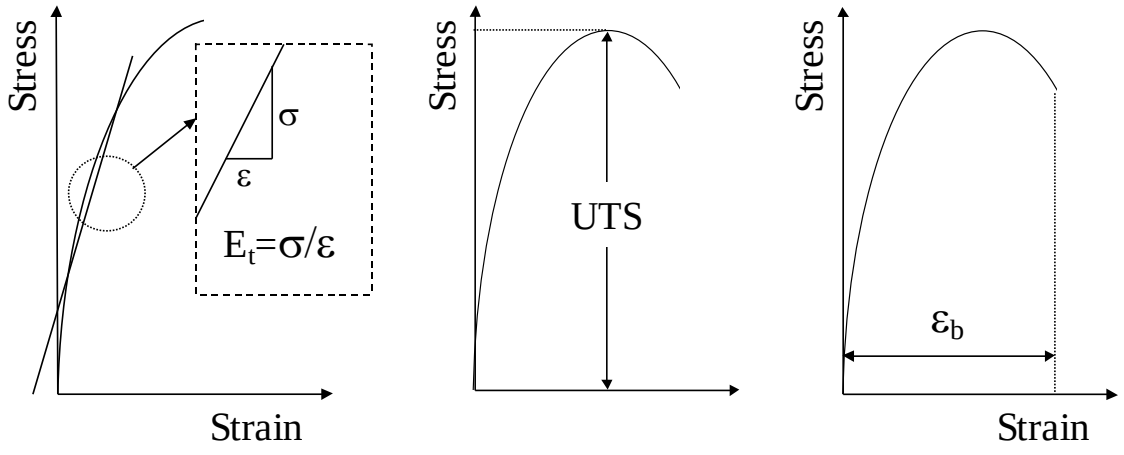


**Figure 2. 8:** Graphical representation of the tangent modulus at 1 % strain, ultimate tensile strength and strain at break point.

### *5.4.2. Compression testing*

3D structures developed in Chapters 6 and 7 were mechanically tested by compression experiments in an Instron 5543 universal testing machine (Instron Int. Ltd., UK) with a load cell of 1kN. Compression testing was carried out at a crosshead speed of 2mm/min, until obtaining a maximum reduction in samples height of 60%. Compressive modulus was determined in the most linear region of the stress–strain graph using the tangent method. A minimum of five samples of each type/condition were tested.

## *5.5. Water uptake and degradation tests*

Water uptake and degradation can be easily quantified by a mass based method. For water uptake, generally, samples are weighted out into screw-top plastic tubes, immersed in phosphate buffered saline (PBS) and allowed to swell at 37ºC, with gentle agitation (60 rpm) in a precision water bath. After each time point, the samples are brought to surface dryness, on a Whatman filter, and weighted.
The content of water in the swollen scaffolds is then calculated by the following equation:

$$Water\ Uptake\ (\%) = [(Ws - Wi)/Wi] \times 100$$

where $w_i$ is the initial weight of the specimen before immersion and $w_s$ is the weight of the swollen structure.

After each time period the samples are dried and weighted to determine the weight loss, which is calculated according to the equation:

$$Weight\ Loss\ (\%) = [(Wd - Wi)/Wi] \times 100$$

where $w_d$ is the final weight of the sample (dried) and $w_i$ is the initial weight of the sample.

## *5.6. In vitro dexamethasone release profile*

In order to quantify the release of a drug from the produced structures, a simple methodology was employed. Drug-loaded structures were immersed in PBS (pH 7.4) and incubated at 37°C for a certain period of time. The release of dexamethasone was periodically monitored by extracting 500 µl aliquots. Retrieved aliquots were replenished with 500 µl of PBS. The concentration of dexamethasone was determined by UV–Vis spectroscopy at 242 nm (Shimadzu UV 1601, Japan). Drug concentration was calculated using a standard curve, relating the concentration of drug with the absorbance intensity.

### *5.6.1. Mathematical modeling of dexamethasone release from ulvan membranes*

Different methods have already been developed to try to model the release of a drug from a delivery system. The advantage of these studies relies on the fact that they help understand the mechanisms of mass transfer behind drug release and predict the effect of the matrix properties on this release kinetics.[71]

In the present research work, and in order to understand the mechanisms of dexamethasone release from ulvan structures the power law was applied to the experimental data. This particular approach was chosen as it accounts for both drug diffusion and matrix swelling.[71] Power law is a simple empirical equation, which describes a linear relationship between logarithm of the percentage of drug released and logarithm of time, up to 60% of the maximum drug released.

$$\frac{M_t}{M_\infty} = kt^n$$

$M_t/M_\infty$ is related with the fractional drug release, $k$ is the kinetic constant, $t$ refers to the release time and $n$ is the diffusional exponent that is related to the drug transport mechanism. According to this law, the release mechanism is determined by the diffusional exponent (Table 2.4). It can be governed by a Fickian Transport, where the release occurs through molecular diffusion of the drug due to a concentration gradient; Case-II Transport, which is mostly associated with swelling, due to the relaxation of the macromolecules upon water uptake into the polymeric matrix or it can be governed by both drug diffusion (diffusional release) and polymer swelling (relaxation release), in a mechanism known as Anomalous Transport.[63, 71]

**Table 2. 4.** Mechanisms behind drug release from polymeric devices, with various geometries, and exponent *n* of the power law equation. Adapted from Siepmann & Peppas, 2001.[71]

| | Film | Cylinder | Sphere | Release Mechanism |
|---|---|---|---|---|
| ***n*** | 0.5 | 0.45 | 0.43 | Fickian Transport (diffusional release) |
| | 0.5<n<1.0 | 0.45<n<0.89 | 0.43<n<0.85 | Anomalous Transport (diffusional and relaxation release) |
| | 1.0 | 0.89 | 0.85 | Case-II Transport (relaxation release) |

### *5.7. Biological assays*

Systemic and physiological material's related toxicity events can be complex and difficult to evaluate, both *in vivo* and *in vitro*.[53] For this reason, toxicity is generally evaluated at the cellular level – cytotoxicity.[53] The basis of cytotoxicity studies employed on the study of biomaterials was already explained in more detail in section 3.8. Briefly, the effect of a material over normal cellular function is evaluated by monitoring of some targeted cellular responses and mechanisms, like metabolism and viability, growth and/or survival, etc.[53]

In the present work, the general guidelines of the ISO/EN 10993 part 5 were followed[45], and cellular metabolic activity and/or proliferation were accessed. Different types of methodologies can be applied in order to access the toxicity of a developed polymer matrix, namely extract and direct and indirect contact assays, followed by the evaluation of different parameters, including cell damage, growth or metabolism.[45]

#### *5.7.1. Cell lines*

As for the evaluation of cytotoxicity of ulvan extracts, mouse C3H/An connective tissue fibroblast-like cells (L929), obtained from the European Collection of Cell Cultures (ECCC, UK), were used for cytotoxicity testing of produced structures.

#### *5.7.2. Cell culture*

Cells were cultured in tissue culture flasks at sub-confluent cell density in Dulbecco's modified eagle medium (DMEM) supplemented with sodium bicarbonate (44 mM) (Sigma–Aldrich, Germany), 10% fetal bovine serum (FBS – Alfagene, USA) and 1% of antibiotic-antimycotic solution (Gibco, UK). Cultures were maintained at 37°C, in a humidified tissue culture incubator with a 5 % CO2 atmosphere. Medium was renewed every 2-3 days.

#### *5.7.3. MEM extract and evaluation of cellular metabolic activity by MTS assay*

MEM extract test is widely employed in the study of cytotoxicity of leachables/soluble products of degradation from biomaterials. For this purpose cells are exposed to a prepared material extract (containing the material's leachables), after which the effect on the cells is measured (growth, metabolism, membrane damage, etc.).

This particular test was performed to assess the cytotoxicity of PDLLA based structures; extracts of the materials were prepared and placed in contact with mouse L929 cells. Short-term cytotoxicity of leachables released from polymeric biomaterials towards L929 cells was evaluated through MTS assay.

In summary, $5x10^3$cells.ml$^{-1}$ were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum and 1% antibiotic/antimycotic solution and allowed to grow until a desirable confluency was reached. At this time, culture media was replaced with material extracts, prepared in culture media. L929 cells viability was determined for each extract and compared to latex extracts, used as a positive control of cell death, and to cells growing in the absence material extracts (negative control, representing cell growth in culture media). After each time point (24, 48 and 72 h of incubation) MTS test was performed to assess cellular viability, following the manufacturer's instructions (details about this test can be found in section 3.8.4.). Optical density was measured at 490 nm, on a multi-well microplate reader (Synergy HT, Bio-Tek, USA).

### *5.7.4. Direct contact: viability and proliferation of cells in the presence of ulvan 3D porous structures*

The choice of a direct contact test in detriment of an extract assay to evaluate the cytotoxicity of these particular structures is largely related with the inherent properties of these structures, namely their water uptake ability (that can be as high as 2000%). This fact *per se* hampers the application of an easy and straightforward MEM extract test. The application of a direct contact test has the advantage of allowing the simultaneous evaluation cellular behaviour upon contact with the prepared structures.

Briefly, confluent L929 cell at passages 20–26 were harvested for seeding onto ulvan samples at a density of $5.0x10^5$ cells/scaffold.[72] The constructs were cultured in standard medium for 1, 3, and 7 days. Cells seeded on standard tissue culture polystyrene were used as a negative control, representing cell normal growth in culture media. In this experiment cell adherent culture plates were chosen. Cells non-adherent to the samples were used, as a non standard method to screen possible toxicity of soluble components and other degradation products resulting from ulvan samples.

The effect of the produced ulvan structures onto L929 cells was evaluated by assaying different parameters through MTS assay, dsDNA quantification and cell imaging. Both cell metabolic activity (MTS assay) and dsDNA quantification were performed on cells cultured on ulvan structures and on cells that end up growing in the culture plate.

- MTS assay

Details about this test can be found in section 3.8.4. In summary, cell viability for each culturing time was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, USA), following manufacturer instructions. Absorbance

of four samples per type of ulvan structure and time point was measured at 490 nm in a microplate reader (Synergie HT, Bio-Tek, USA). Cell proliferation was quantified by the total amount of double-stranded DNA (dsDNA), along the culturing time.

- dsDNA quantification

Details about this test can be found in section 3.8.4. Quantification was performed using PicoGreen dsDNA Assay Kit (Invitrogen™, Molecular Probes™, USA), according to the manufacturer's instructions. Briefly, cells were lysed by osmotic and thermal shock and the supernatant used for the DNA quantification assay. Fluorescence was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, in a microplate reader (Synergie HT, Bio-Tek, USA). Quadruplicates were made for each sample and per culturing time. dsDNA concentration for each sample was calculated using a standard curve relating DNA concentration (ranging from 0.0 to 1.5 mg/mL) and fluorescence intensity.

- Cell Imaging

Viability, morphology and distribution of cells on ulvan structures were assessed, after 1 and 3 days, by reflected/transmitted light microscope, following calcein-AM and propidium iodide staining, and by scanning electron microscopy (SEM).

For reflected/transmitted light microscope visualization, ulvan structures were incubated in culture medium with calcein-AM and propidium iodide (Invitrogen™, Molecular Probes™, USA). These fluorescent markers are used to label cells and allow visualization by selecting an appropriated wavelength.[53] Calcein-AM is a non-fluorescent permeable compound that once inside viable cells is converted by intracellular esterases into a fluorescent cell impermeable form, being used as a green fluorescent indicator of viable cells. This particular marker does not label dead cells, and is rapidly lost under conditions that cause cell lysis. In order to visualize dead cells, propidium iodide staining was performed. Propidium iodide is used as a red fluorescent marker of cells with compromised membranes, being used as an indicator of dead cells. The calcein-AM/propidium iodide stained samples were placed on a microscope slide and observed by reflected/transmitted light microscope (Zeiss, Axio Imager.Z1m, Germany).

For SEM analysis (FEI Nova 200 SEM, FEI, USA), ulvan structures were fixed with 2.5% glutaraldehyde. This fixation step is necessary for stabilization of the overall structure (cells on the matrix), allowing the maintenance of the biological samples in

their natural state. Structures were further dehydrated in increasing ethanol concentrations, critical point dried and gold sputter coated prior to SEM observation (details about SEM are further reviewed in section 5.2.).

## 6. Statistical analysis

Statistical analysis was performed using the GraphPad Prism statistic software (Release version 5 for MS Windows).

In a first approach, a Shapiro-Wilk test was used to ascertain about data normality.[73] As data failed to demonstrate a normal distribution, non-parametric tests were performed for further comparisons.

Whenever two independent samples on one variable needed to be compared, a Mann-Whitney test was applied.[74] This particular test was employed for:

- study of ulvan's polysaccharide cytotoxicity - direct comparisons between ulvan and hyaluronic acid (Chapter 4);
- study of 3D porous structures produced by freeze drying - direct comparison between two structures with varying polysaccharide content (Chapter 6);
- Study of the effect on mechanical performance of the presence of ulvan particles within a PDLLA matrix (Chapter 7).

For the analysis of more than two independent groups of samples compared for one variable, a Kruskal-Wallis test was employed.[75] When this test indicated significant differences between independent groups, different multiple comparison tests were performed to identify different groups. The Dunn's test is a specialized multiple comparison test that allows to compare a single control group to all other sample groups. These tests were generally applied to study the effect of the test samples (different polysaccharide concentrations, both of ulvan and hyaluronic acid, ulvan porous structures and PDLLA based matrix enriched with ulvan particles) over various cellular biochemical parameters, namely cellular metabolic activity and cellular number and proliferation.

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# SECTION III – EXTRACTION OF A VALUABLE SULPHATED POLYSACCHARIDE FROM GREEN ALGAE *ULVA LACTUCA* AND ITS CHARACTERIZATION

# Chapter 3

## Extraction and physicochemical characterization of a versatile biodegradable polysaccharide obtained from green algae

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This chapter is based on the following publication: Alves A, Caridade SG, Mano JF, Sousa RA and Reis RL, Extraction and physicochemical characterization of a versatile biodegradable polysaccharide obtained from green algae. 2010. Carbohydrate Research, 345 : 2194-2200.

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## Abstract

During the last years, considerable attention has been given to different marine organisms, like algae, as potential sources of valuable materials. The continuous demand for novel materials and technologies is high and research on the underexploited marine green algae, including its polysaccharidic part - ulvan, has increased accordingly. In this research work, a novel method for extraction of ulvan from green algae is proposed and demonstrated successful. Different characterization techniques were employed to characterize the isolated algal polysaccharide, namely on what concerns its thermal trace and crystallinity. Upon heating, ulvan behaves as a non-meltable polysaccharide that is thermally stable before degradation at 220$^{o}$C. Ulvan is semi-crystalline in nature and possesses high hygroscopic features, as revealed in this research work. Due to its properties, ulvan can be considered, pure or modified, as a versatile biodegradable polymer for different applications, including tissue engineering and regenerative medicine.

## 1. Introduction

Natural polymers are becoming more and more important for technological and industrial related applications. These are believed to be a better alternative approach to the use of fully synthetic materials in different contexts.[1] Nowadays, a lot of the research effort in the biomedical field, namely tissue engineering, is based on natural polymers.[2-4] Among these, polysaccharides are at the centre of attention as they possess unique physical and chemical properties, being recognized not only for their important functional properties but also for their potential biological applications. The list of known natural carbohydrates is continuously growing, owing to new discoveries in animal and plant material.[5] They can be used in their native form or after chemical modification, as is the case of chitin or cellulose, in order to render them suitable for the envisaged applications.[6]

Over the last years, different marine organisms have been raising considerable interest as sources of valuable materials. Algae, for instance, has been target of special attention. The unique properties of polysaccharides that are found in these organisms are especially interesting, which emphasizes the importance of obtaining further knowledge in this area. In fact, the exploitation of algae for different applications and as a source of carbohydrate polymers is not a novelty as these polymers have long been used. Nevertheless, among the three main divisions of macroalgae (Chlorophyta, Phaeophyta and Rhodophyta), green algae remain largely unexploited. In the past decade, marine eutrophication has promoted the proliferation of algal biomasses, namely of *Ulva* sp. This is associated with an increase in human activity in coastal areas, which potentiates an increase in some nutrients in the aquatic environment, leading to growth of green algae, commonly known as algal blooms. As a consequence, the interest in exploiting such natural resource has increased significantly and, in particular, for its polysaccharidic part, ulvan.[7] This designation is used to denote polysaccharides from members of the Ulvales algae, namely *Ulva*. Ulvan is mostly composed of rhamnose, glucuronic acid, iduronic acid, xylose, and sulphate.[8] To extract ulvan from green algae, different methodologies have been employed[9-12], based on the solubility of this polysaccharide in water. However, a special concern must be given to purification, as it will be of outmost importance to the final use of this polymer. In this context, a simple and novel extraction procedure is proposed. An important input and insight on ulvan's properties has been already given by many research works.[8-10, 12-16] Still, many of its characteristics and properties remain unknown, namely on what concerns its thermal trace and crystallinity. The precise knowledge of thermal behaviour and crystallinity of this polysaccharide assumes a

crucial role on its characterization, as different properties, such as mechanical performance, solubility, among others, are expected to be directly influenced by these features.

In this study, some of the functionalities of this polysaccharide are unveiled and a simple method of extraction of ulvan is proposed.

## 2. Results

### *2.1. Chemical characterization of the extracted polysaccharide*

A novel yet simple extraction procedure was defined in order to obtain a final extract suitable for different applications, including food and biomedical manufacturing processes. Extraction methodology was carried out as schematically represented in Figure 3.1. The yield of this procedure is c.a. 10-20%.

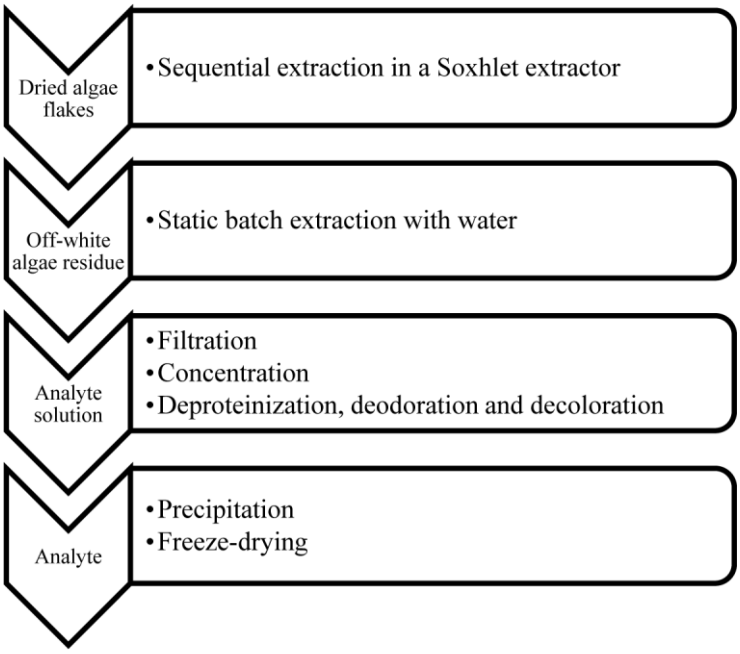


**Figure 3. 1.** Scheme for the extraction of water soluble material, namely ulvan, from green algae.

Elemental analysis, presented in Table 3.1, revealed the presence of proteins and sulphate. These results are in agreement with the fact that the polysaccharide ulvan is a sulphated polysaccharide present in the cell wall of green algae, in close association with proteins, as described by Lahaye and Robic.[8, 17]

**Table 3. 1.** Elemental analysis of ulvan extracted from green algae collected at the portuguese coast.

| | % N | % C | % H | % S | Protein (%) |
|---|---|---|---|---|---|
| **Ulvan a** | 1.50±0.01 | 20.55±0.09 | 3.23±0.01 | 9.07±0.58 | 9.37 |
| **Ulvan b** | 1.58±0.01 | 20.20±0.13 | 3.29±0.06 | 6.98±1.95 | 9.87 |

On the other hand, we have compared the chemical elements present in two different batches of ulvan, in order to account the possible variability associated with the extraction of natural compounds. In our study, despite the existence of variability we can observe that the two batches of ulvan are similar. The amount of chemical elements and molecules, like proteins and sulphate may vary for different reasons, namely methodological, ecophysiological and taxonomical reasons. Nonetheless, these results are in agreement with past findings on the elemental characterization of ulvan.[11, 18]

In order to further characterize ulvan extracted from green algae and to identify the fundamental groups present in its structure, FTIR analysis was performed. The infrared spectrum of this polysaccharide is given in Figure 3.2.

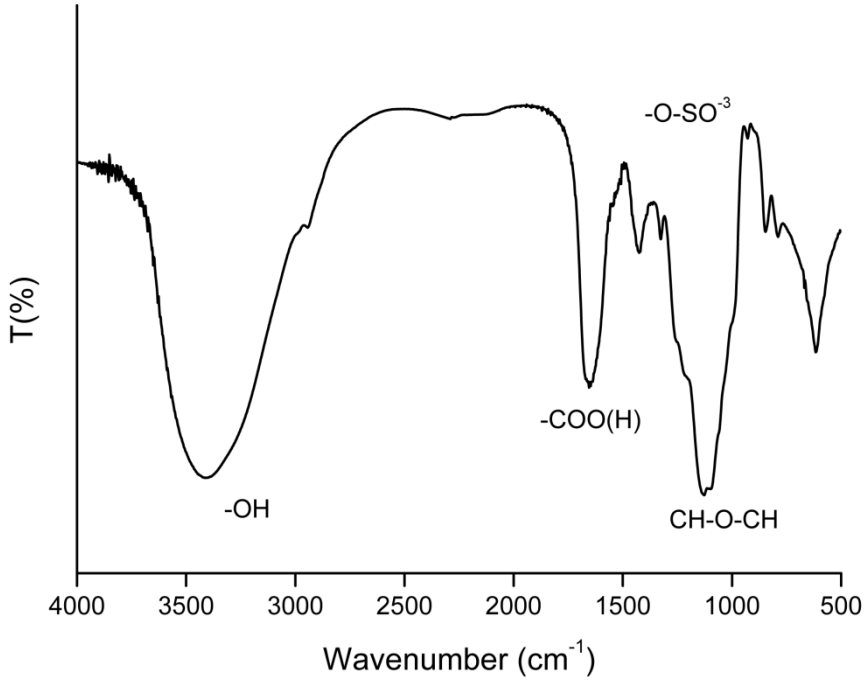


**Figure 3. 2.** Infrared spectrum of ulvan between 400 and 4000 $cm^{-1}$. The main characteristic functional groups are depicted in the graph.

Stretching of polymeric hydroxyl groups is identified by a broad band in the range 3500–3200 $cm^{-1}$. The presence of a carboxylic acid is revealed by a band at ~ 1650 $cm^{-1}$. This carbonyl group probably is in the form of a salt (carboxylate).[5] The asymmetric stretching of the ether glycosidic bridge (CH–O–CH) is shown by an intense band around 1130$cm^{-1}$. The signals in the ranges of 1315–1220$cm^{-1}$ and 1140 –1050$cm^{-1}$ are assigned to the symmetric and asymmetric stretching of an ether sulphate group (RO–$SO^{-3}$).

$^{1}$H-NMR spectroscopy is a fundamental tool when studying the chemistry of polysaccharides. However, due to the high number of possible disaccharide sequencings and distribution in addition to other possible structural irregularities, obtained spectra are many times intricate, which enhances the complexity of analysing the signals.[8, 19] However if one is not intending quantification this technique can be used to identify the presence of sugar moieties, like iduronic acid. The choice of this technique over others relies on some of the relative advantages of NMR, especially on what concerns simplicity of sample preparation, easier calibration of the equipment, faster optimization of experimental protocols and less time required for experimental results. Despite the complexity of carbohydrate NMR spectra, information can be obtained about the chemical composition of the extracted polysaccharide.[19-20] Proton NMR spectrum of extracted water soluble polysaccharide is represented in Figure 3.3.

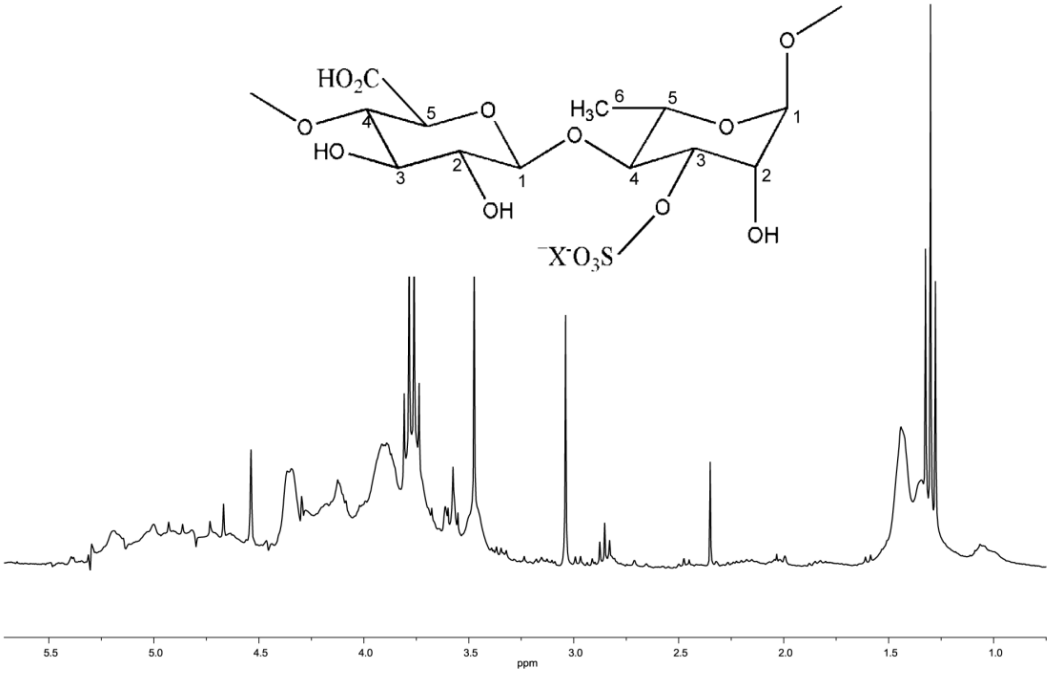


**Figure 3. 3.** $^{1}$H-NMR spectrum of ulvan, at 25ºC.

Proton signals were assigned taking into account the chemical shifts of mono and oligosaccharides of ulvan[21] and methylation analysis[14, 22] information. Table 3.2 shows the proton chemical shifts associated to each carbon atom present in the molecule. Accordingly, anomeric proton chemical shifts of iduronic acid and rhamnose 3-sulphate linked to iduronic acid are 5.00 and 4.92, respectively. The proton chemical shifts attributed to rhamnose are 4.86, 4.30, 4.52, 3.60, 4.12 and 1.30. Characteristic glucuronic acid chemical shifts are 4.73, 3.47, 3.72, 3.72 and 3.80. Iduronic acid proton chemical shifts are 5.02, 3.72, 3.82, 4.09 and 4.66.

**Table 3. 2.** Ulvan proton chemical shifts assignments obtained through the analysis of $^{1}$H-NMR spectrum.

| | $C_1$ | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $C_6$ |
|---|---|---|---|---|---|---|
| **Rhamnose** | 4.86 | 4.30 | 4.52 | 3.60 | 4.12 | 1.30 |
| **Glucuronic Acid** | 4.73 | 3.47 | 3.72 | 3.72 | 3.80 | - |
| **Iduronic Acid** | 5.02 | 3.72 | 3.82 | 4.09 | 4.66 | - |

### *2.2. Physical properties of extracted ulvan*

The thermal properties of ulvan extracted from green algae were investigated by means of thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) and dynamic mechanical analysis (DMA).

Thermogravimetric analysis provides a quantitative measurement of mass change in materials associated with dehydration, decomposition and oxidation of a sample with time and temperature. Ulvan characteristic thermogravimetric curve is presented in Figure 3.4.

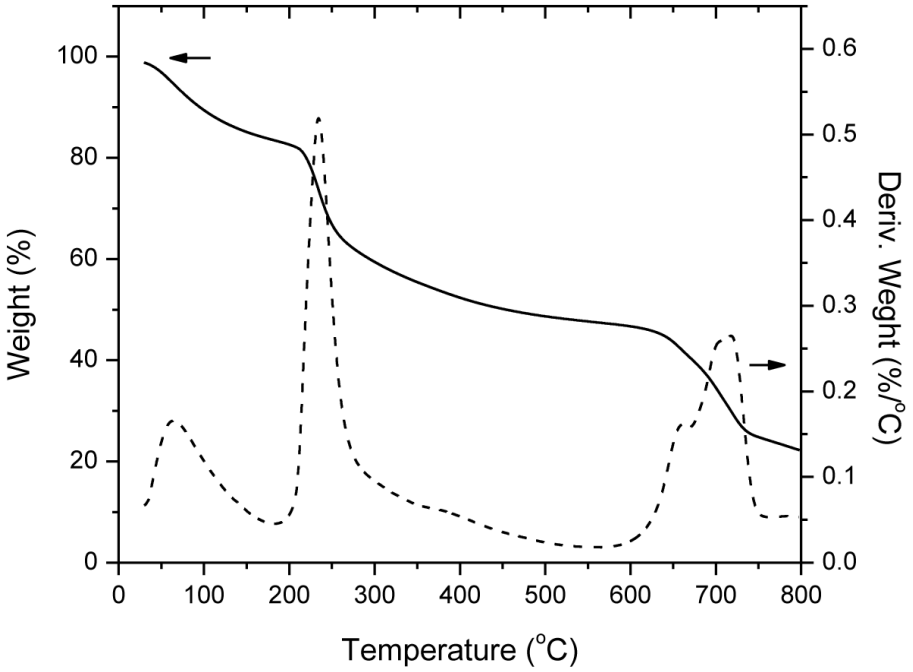


**Figure 3. 4.** Characteristic thermogravimetric curve of ulvan, recording mass loss versus increasing temperature, with its first derivative.

The initial mass loss is related to the volatilization of moisture, as hydrogen bound water, present in the sample, associated with desorption of bound water and dehydration reactions. Moisture associated with ulvan is around 20%. The first polysaccharidic decomposition step starts at around 220ºC. The second decomposition step at ~700ºC can be attributed to inorganic material present in the sample. The total content of ash is estimated around 20% of the initial weight of the sample. This high amount of ash can be attributed to counterions associated with sulphate groups and uronic acids of ulvan.[14, 23] On the other hand, TGA is primarily used for determination of thermal stability of a sample. In this case, one can ascertain ulvan's thermal stability until c.a. 200°C.

In order to identify thermal transitions of ulvan, differential scanning calorimetry was employed. The thermal performance of this polysaccharide is depicted in Figure 3.5.

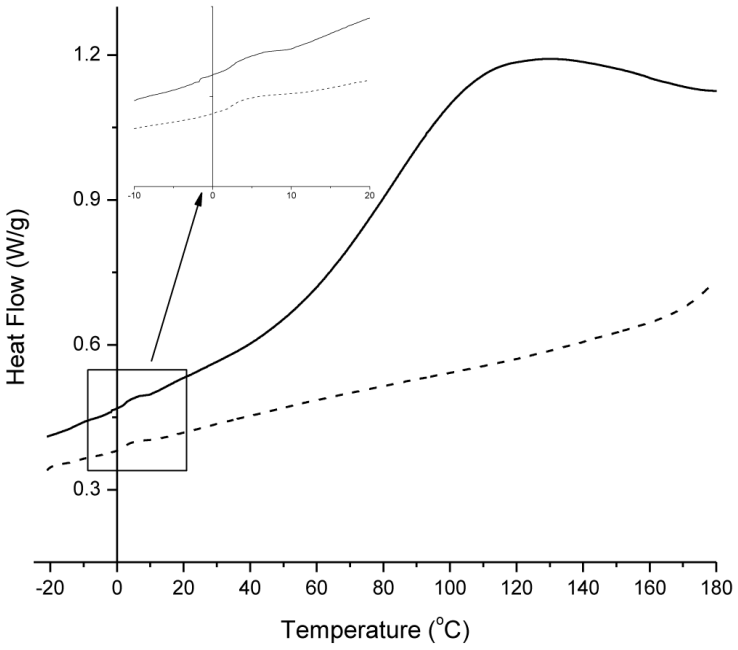


**Figure 3. 5.** Thermogram of ulvan, on heating: first heating scan (straight line) and second heating scan (dash line) (Endo up).

Two transitions were detected in the samples scanned in a temperature range of -20°C to 180°C. The major transition is associated with a large endothermic peak detected at about 120ºC. This peak is not detected in the second run and therefore can be attributed to absorbed moisture (water), which is in agreement with the data recorded in TGA. The observation of this endothermic peak has already been reported by different authors for various polysaccharides.[24-25]

A less pronounced thermal transition is observed at 5ºC. This shoulder in the DSC trace is still detected in the second run and the nature of this low temperature transition is still not understood. It may be associated with a small quantity of ice, which is in agreement with the presence of bound water. As in many unmodified polysaccharides, like chitosan[26], ulvan undergoes thermal degradation prior to melting, as observed in a melting point meter (data not shown).

The presence of the glass transition in ulvan is not clear or not observed in this DSC study. In some cases, differential scanning calorimetry may not be sensitive enough to detect the relaxation temperature of semi-crystalline polysaccharides. Consequently and in order to further understand the thermal transitions detected in DSC experiments, dynamic mechanical analysis was performed. Until recently, DMA was only used to study self-supporting materials. However, it is now possible to apply this technique in

the study of powders[27] and in order to effectively detect their thermal transitions, like glass transition. Figure 3.6 shows the resulting thermogram for ulvan at 1Hz.

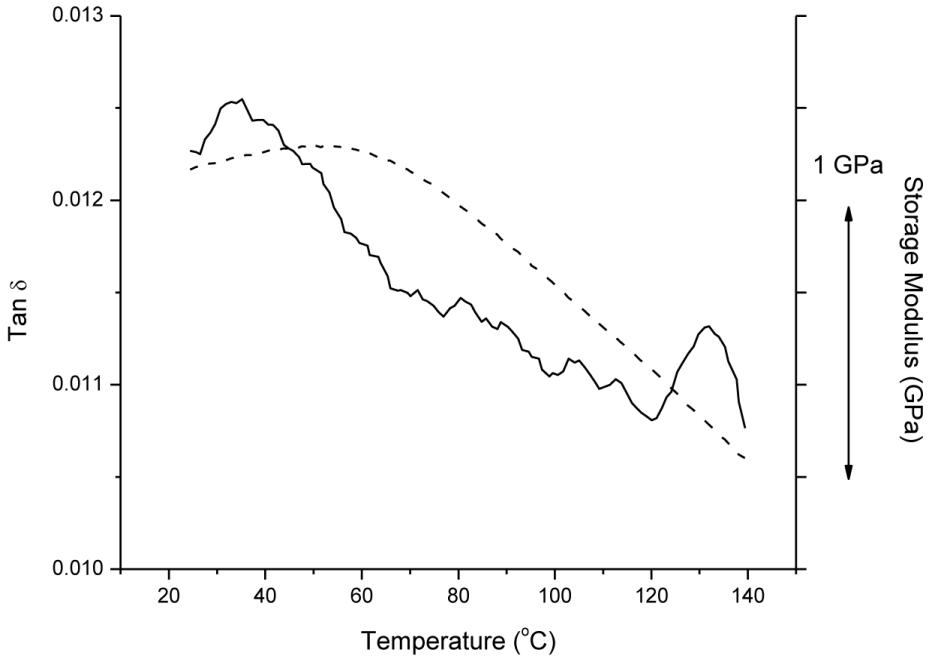


**Figure 3. 6.** Ulvan thermogram obtained from DMA, at 1Hz: tan δ (straight line) and storage modulus (dashed line).

It can be observed a peak for tan *δ* at around 30ºC and it may be related with the low-temperature transition, previously identified by DSC (around 5ºC). As DSC and DMA measure different processes, the difference in readings can be of as much as 25ºC in data. Another thermal transition was also detected at around 130ºC. We believe that this is associated with water loss and it is in agreement with previous findings reported by the TGA and DSC studies.

XRD analysis was applied to determine the crystallinity degree of extracted ulvan. The XRD pattern of this polysaccharide, presented in Figure 7, is typical for a semi-crystalline polymer and shows five major crystalline reflections at 13.1$^{o}$, 23.2$^{o}$, 26.4$^{o}$, 32.6$^{o}$ and 39.4$^{o}$.

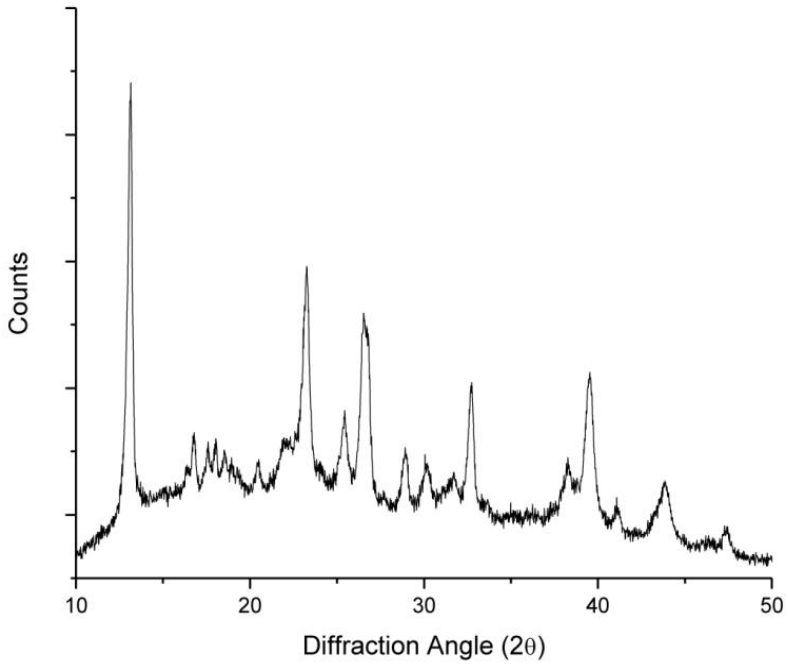


**Figure 3. 7.** X-ray diffractogram of ulvan powder.

## 3. Discussion

A novel procedure was studied and applied to extract water soluble ulvan from green algae. Different procedures were described in the literature for the extraction of sulphated polysaccharides from green algae.[7, 12-13, 17, 28] According to the intended application of the final extracted polysaccharide, different processes must be used depending on the envisaged purity degree of the polymer, which is especially relevant in the case of human related applications. Chemical characterization by chemical analysis, infrared and $^{1}$H-NMR spectroscopy indicate that the structure of the obtained acidic sulphated polysaccharide is similar to that of ulvan already reported in the literature. The extracted water soluble polysaccharide IR spectrum (Figure 3.2) is comparable with the obtained spectra of ulvan.[14, 29] This fact demonstrates that the proposed extraction process is able to maintain ulvan structure and provides a good indication that its intrinsic properties are also preserved. FTIR (Figure 3.2) and $^{1}$H-NMR (Figure 3.3) results also reveal the polysaccharidic nature of the obtained water soluble ulvan, as well as the presence of rhamnose and uronic acids, with sulphate groups, as previously found for ulvan. The molecular structure of the extracted polysaccharide can therefore be inferred as the one already proposed for ulvan and it is depicted in Figure 3.8.[21]

On the other hand, and accounting with the variability associated to the extraction of natural origin molecules, we can observe similarity within different ulvan batches extracted with the method proposed in the present study.

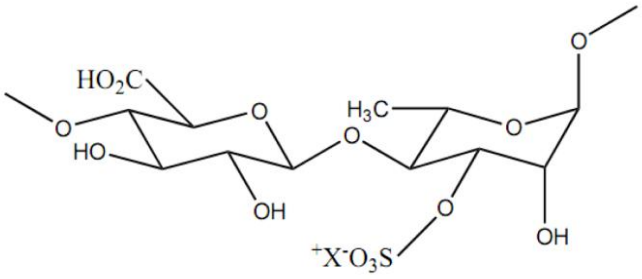


**Figure 3. 8.** Molecular structural motif constituent of ulvan.

Besides chemical characterization, physical properties are relevant to establish the processability of a polymeric material and their potential use in consumer oriented applications. For example, in many preparation and processing methodologies, polysaccharides must be submitted to high temperatures. In this context, thermal characterization is an important step for the study of thermal transitions, which may reveal important information concerning the adequate temperature intervals compatible with the processing of materials based on ulvan. Up to date, there are no reported results focusing the thermal events occurring in this polysaccharide.

Thermogravimetric analysis is a simple and accurate method for studying the decomposition pattern and the thermal stability of polymers. Parikh described, in 2006, thermal degradation of polysaccharide as a four step process:

(1) Desorption of physically absorbed water;

(2) Removal of structural water (dehydration reactions);

(3) Depolymerisation accompanied by the rupture of C-O and C-C bonds in the ring units resulting in the evolution of CO, $CO_2$ and $H_2O$;

(4) Formation of polynuclear aromatic and graphitic carbon structures.[30]

According to the thermogravimetric present results (Figure 3.4), ulvan undergoes thermal degradation in a very typical manner. On the other hand, ulvan is thermally stable until c.a. 200°C. Villetti and co-workers studied the thermal degradation mechanisms of different natural polymers and observed that polyanions, like sodium hyaluronate and xanthan, possess low thermal stability in comparison with neutral polysaccharides, like methylcellulose.[31]

To further understand the thermal behaviour and transitions occurring in ulvan, DSC was performed. Ulvan thermogram in Figure 3.5 exhibits a major transition around 120ºC. A peak in tan *δ* was also detected by DMA in this temperature range, at around 130ºC (Figure 3.6). This transition could be associated with loss of water, being consistent with the hydrophilic nature of ulvan functional groups; the presence of this peak can also reveal the existence of bound water.[32-33] Morelli and co-workers have identified this event as the glass transition of ulvan.[34] However, in the present case, a vitreous transition should have been observed during a second heating run, which does not occur. Based on these findings, such event will not be regarded as a glass transition.

A less pronounced thermal transition is observed at 5ºC by DSC (Figure 3.5) that could also be associated to the process detected by DMA at 30ºC (Figure 3.6). In order to better understand this thermal event, the Arrhenius equation was applied to calculate its activation energy.

$$lnf = lnA - \left[\frac{E_a}{R \cdot T}\right]$$

*f* is the applied frequency, *A* is a constant, *R* is the universal gas constant and *T* is the peak maximum temperature of the tan *δ* peak. Plotting the natural logarithm of the frequency against the inverse of the observed peak temperature gave a linear relationship with a regression coefficient of 0.98 (data not shown) and an activation energy of 248 kJ/mol. Such value is in the order of the activation energy found for the glass transition of polymeric systems.[35] However, the low intensity of the tan *δ* peak suggests that this process should occur in a small fraction of the analysed polymeric system, for example, due to the presence of some impurities in the sample, and may not be related to the polysaccharide itself. In light of the present results, both DSC and DMA experiments did not allow the identification of a clear occurrence of a glass transition in ulvan in the temperature interval analysed. Many times, the direct identification of a glass transition temperature in polysaccharides can be a delicate task, mostly because the temperature range at which the main relaxation occurs is close to the degradation temperature of the material, as is the case of amorphous cellulose.[36] Other factors as water-polysaccharide interactions can also increase complexity in identifying glass transition in polysaccharides. Nonetheless, thermal behaviour and the associated reactions occurring over specific temperature ranges and heating rates represent a unique sequence for each material. The distinctive characteristics unveiled in this work represent ulvan thermal performance, which are believed to be intimately related to its molecular structure.

From the the x-ray diffractogram of ulvan (Figure 3.7), the semi-cristalline nature of this polysaccharide was revealed. This indicates that the amorphous component of ulvan dominates over the crystalline organized one. This structural arrangement directly affects different properties such as tensile strength, flexibility, solubility, swelling or opaqueness of the bulk polymer, as physical properties are dependent on the degree of order within the material.[36]

Ulvan is water soluble and hygroscopic hydrocolloid, as seen by the presence of bound water and due to the hydrophilic nature of some of its functional groups. This property is also related with the semi-crystalline nature of this polysaccharide. In fact, the crystalline structure includes a fixed number of water molecules, while the amorphous zones accommodates an increasing amount of moisture depending on the water activity of the atmosphere where the samples are being equilibrated.[37] This particular feature is an important character for many consumer oriented applications, as in food industry, or to improve polymer processing.

A table summarizing ulvan's properties is depicted in Table 3.3.

**Table 3. 3.** General properties of ulvan extracted from green algae.

| **Properties** | |
|---|---|
| Functional Groups | -COO(H), -OH, -$OSO_{(4)}$ |
| Colour | White |
| Protein | cc. 9 – 10% |
| Sulphate | cc. 9 – 10% |
| Moisture | cc. 20% |
| Ash | cc. 20% |
| Solubility | Water Soluble |
| Hygroscopy | Hygroscopic |
| Thermal Stability | Until cc. 220$^{o}$C |
| Melting Temperature | Non-meltable |
| Glass Transition | Not detected |
| Crystallinity | Semi-crystalline |

Ulvan can be considered for itself as a versatile and promising biodegradable polymer to be used in different areas, ranging from health care to different industrial applications. Its functional properties are expected to be improved by physical or chemical modifications, like complexation with other polymeric materials, chemicals or salts or modifications introduced in the polysaccharidic chain.

## 4. Conclusions

The scope of this paper was twofold. First, the main goal was to establish a new and simple method of extracting ulvan from green algae. Secondly, the work herein presented was carried out in order to gain a better understanding on the extracted polysaccharide ulvan, in order to define its potential applications. However, apart from the relevance of any fundamental study of polysaccharides, this study contributes to unravel the physical properties of ulvan and its technical use. Up to date, there are no reported results regarding the thermal transitions occurring in the polysaccharide ulvan and information related with its crystallinity. In the present study, it was possible to conclude that ulvan is a non-meltable semi-crystalline polysaccharide, which is thermally stable before degradation at 220 ºC.

## 5. Materials and methods

### *5.1. Extraction of ulvan polysaccharide*

Soxhlet extraction of dried *Ulva* sp. with organic solvents removed most of the lipids and colouring matter which otherwise would contaminate the water extract. The residual off-white weed was dried and subjected to three hot water extractions, each with c.a. 0.5l, for a total of 8h at 75-85ºC, on a boiling-water bath, under continuous stirring. After filtration through a cotton cloth, the aqueous extracts were centrifuged, and the liquid supernatant was filtered. The water extract was concentrated until 10-20% of its initial value, in a rotary evaporator. The derived solution was then deproteinized by proteinase digestion, with proteinase K. Afterwards, the solution was decolorized and deodorized by adsorption on activated charcoal. The water extract was centrifuged, filtered and precipitated with 4 vol. of absolute ethanol. Finally, the recovered precipitate was freeze dried. Yield of a white polysaccharide resulting from this extraction methodology is c.a. 10 – 20%.

### *5.2. General methods*

Elemental analysis (% C, H, N and S content) was performed by combustion to evaluate the content of the major components present in the extracted analyte. A Carlo Erba CHNS-O EA 1108 apparatus was used.

Proteins were measured as nitrogen content through Kjedhal analysis.[38] According with this method, it is possible to calculate the amount of crude protein (*CP*) in the sample by multiplying the percent nitrogen (*N*) by a conversion factor (6.25), through the following equation.

$$CP = N \times 6.25$$

To assess the chemical constituents of the extracted polysaccharide, powder was analyzed by infrared spectroscopy (FTIR) in transmission mode using a IR Prestige-21 apparatus (Shimadzu, Japan). Pre-dried powder was mixed with KBr and then formed into a disc by manual pressing. Transmission spectra were recorded using at least 32 scans with 4 $cm^{-1}$ resolution in the spectral range of 4,000 – 400 $cm^{-1}$.

$^{1}$H-NMR spectrum of pre-dried polysaccharide dissolved in $D_2O$ was recorded on a Varian Unity Plus spectrometer (Varian, USA), at 25ºC.

For TGA, a small amount (15-18mg) of powder was taken for analysis in a Perkin Elmer TGA7 (Perkin Elmer, USA). The samples were heated from 40 to 720°C at a rate of 10°C $min^{-1}$ in nitrogen atmosphere.

DSC experiments were carried out using TA-Q100 equipment (TA Instruments, USA). The experiments were conducted, under a nitrogen atmosphere, on samples (5-10mg) packed in aluminium pans. The samples were heated in two stages at a constant heating rate of 20ºC/min from -20$^{o}$C up to 180ºC, then were left at this temperature for a period of 2 min and cooled at -20ºC/min to the initial temperature. At this point a second heating run was conducted. An empty aluminium pan was used as reference.

In the present work, DMA (TRITEC 8000B DMA apparatus, Triton, UK) was used to investigate the viscoelastic properties of the analyte. Stainless steel pockets, provided by the DMA manufacturer, for these specific measurements (length = 15mm, width = 7.5mm, thickness = 1 mm) were used to fix the powder material into the DMA loading frame. Such pocket devices are especially useful to analyse powder materials and more details about the protocols for their use can be found elsewhere.[27] The pockets filled with the sample were subjected to sinusoidal loading under Single Cantilever Bending mode conditions. The load was automatically adjusted in order to achieve an imposed displacement equal to 0.05mm. The measurements were performed at frequencies of 1, 5, 7.5 and 10Hz over the temperature range between 20°C to 140°C at 2°C/min at each frequency. Note that the storage modulus that is given by the apparatus is basically resulting from the properties of the metallic component. However, any change in the viscoelastic properties of the sample will be detected in the tan $\delta$ trace.

X-ray diffraction (XRD) patterns were measured using a powder diffractometer Philips PW1710 apparatus (Philips, The Netherlands) operated in step scan mode (1.542Å wavelength). The diffraction data was acquired at a rate of 0.02º $2\theta$/s and over a Bragg angle range of 10º<$2\theta$<50º.

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# Chapter 4

## *In vitro* cytotoxicity assessment of ulvan, a polysaccharide extracted from green algae

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This chapter is based on the following publication: Alves A, Sousa RA and Reis RL, *In vitro* cytotoxicity assessment of ulvan, a polysaccharide extracted from green algae. 2012. Submitted.

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## Abstract

Sustainable exploitation and valorisation of natural marine resources represents a highly interesting platform for the development of novel biomaterials, with both economic and environmental benefits. In this context, toxicity data is regarded as a crucial and fundamental knowledge prior to any advances in the application development of natural derived polymers. In the present work, cytotoxicity of ulvan extracted from green algae *Ulva lactuca* was assessed by means of standard *in vitro* cytotoxicity assays. Fibroblast-like cells were incubated in the presence of this green algae's polysaccharide and cell viability was assayed through 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) test. In addition double stranded DNA and total protein were quantified in order to assess cell number. In order to establish ulvan's non-cytotoxic behaviour, the effect of this polysaccharide on cellular metabolic activity and cell number was directly compared to hyaluronic acid, used as a non-cytotoxic control material. In this study, ulvan demonstrated promising results in terms of cytotoxicity, comparable to the currently used hyaluronic acid, which suggests that ulvan can be considered as non-toxic in the range of concentrations studied.

## 1. Introduction

Living organisms are rich in carbohydrates. The wide chemical and functional diversity of these molecules is related with a rather large source base, which ranges from microbial to animal origin. Carbohydrates that form the structural basis of algae represent a unique group of gel-forming and viscous polymers, which are neither found in higher plants nor digestible by non-ruminant animals.[1] Industrial and technological applications of these marine carbohydrates include, among others, food industry, aesthetics or biomedical devices. In fact, the use of natural origin polymers on the development of novel biomaterials has already proven its feasibility and applicability, particularly in medical related areas, including tissue engineering applications.[2-6]

Being largely unexploited, green algae possess remarkable potential for biotechnological development. Ulvan is a sulphated polysaccharide present in the cell wall of these algae and it is mostly composed of rhamnose, glucuronic acid, iduronic acid and xylose.[7-9] Regardless of the properties of this polysaccharide[10-15], the lack of adequate data on the safety of ulvan, as that derived from standard toxicological studies, limits its applicability as a consumer oriented product. This is especially relevant when biomedical applications are envisioned, such as the use of ulvan as a biomaterial for tissue engineering and regenerative medicine purposes. Furthermore, and apart the study of physicochemical features, evaluation of the cytotoxic performance of any emergent polysaccharide extracted from a natural resource represents a crucial step in its research and development. In this context, biocompatibility assessment must be performed in order to attain information on ulvan's cytotoxicity and appraise its application potential in sectors where consumer oriented applications are envisaged. This is especially important, as ulvan's intrinsic properties may justify its application, in some industrial areas, without high levels of manipulation or significant modification of its native structure. One notable characteristic of ulvan is the presence of rare sugars within its backbone, namely sulphated rhamnose and iduronic acid. The presence of iduronic acid is unusual as this sugar residue has never been identified in algal polysaccharides and it is an important constituent of mammalian glycosaminoglycans, including heparin and chondroitin sulphate.[16-17] Furthermore, the presence of sulphate groups and the unusual chemical composition and structure of ulvan grants different biological properties to this green algae's polysaccharide.[11, 18-26] In this context, it is easy to envisage the use of this polysaccharide in areas such as the pharmaceutical or the biomedical one.

*In vitro* cytotoxicity procedures have proven to be effective in the screening of human chemical toxicity.[27] In standard cytotoxicity tests, the sample is incubated in direct

contact with a layer of cells, usually from a cell line, for a period of at least 24 hours, side by side with positive and negative controls.[28] This is considered an effective method to evaluate and predict the cytotoxicity and the behaviour of a material in the body, since there is no barrier between the material in study and the cell layer.[27] The effect of the presence of a foreign agent on cellular homeostasis may be detected by the disturbance of normal cellular biochemical functions; this knowledge is the basis of many standard toxicological studies.[29-30] In this regard, available commercial assays are based on the measurement of different biochemical functions like the double-stranded DNA quantification (dsDNA), and protein content, through total protein quantification, in order to quantify cellular proliferation; metabolic viability can be determined by mitochondrial activity, through MTS assay, and membrane integrity, assayed through lactate dehydrogenase assay.[30] Loss of viability, reduced proliferation rate or cell membrane rupture represents critical consequences generated by a toxic biomaterial.[31]

In order to assess the intrinsic cytotoxic behaviour of ulvan extracts, different standard cytotoxic strategies were followed, including the evaluation of cellular viability by MTS test and number, through dsDNA and total protein quantification. Furthermore, biological performance of ulvan is evaluated and compared to hyaluronic acid. This polysaccharide is used as a non-cytotoxic control material and it is a well established polysaccharide used in diverse industrial applications, including the biomedical field.[32]

## 2. Materials and methods

### *2.1. Materials*

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Germany). Ulvan was extracted from the green algae *Ulva lactuca*. Unless otherwise stated, these algae were supplied by Setalg (France) as dry flakes with a moisture content ≤15%. Uvan was obtained by extraction from *Ulva lactuca* using a procedure described by the authors elsewhere.[33] In summary, Soxhlet extraction of dried *Ulva lactuca* removed most of the lipids and colouring matter. The residual off-white weed was dried and subjected to three hot-water extractions, between 75-85ºC. After filtration through a cotton cloth, the aqueous extracts were centrifuged, and the liquid supernatant was filtered. The water extract was concentrated until 10-20% of its initial value, in a rotary evaporator. Starch and proteins were removed by enzymatic digestion. Afterwards, the solution was decolorized and deodorized by adsorption on activated charcoal. The water extract was centrifuged, filtered and precipitated with 4 vol. of absolute ethanol.

Finally, the recovered precipitate was freeze dried. Yield of a white polysaccharide resulting from this extraction methodology is c.a. 10 – 20%.
Characterization of the extracted polysaccharide revealed different physicochemical properties which are summarized in Table 4.1.[34]

**Table 4. 1.** According to Costa and co-authors[34], properties and composition of ulvan extracted from the green algae *Ulva lactuca* can be summarized as follows.

| **Ulvan extracted from *Ulva lactuca*** | |
|---|---|
| **Molecular weight** | 790 kDa |
| **Protein** | 1.3% |
| **Sulphate** | 32.2% |
| **Sulphate free inorganic material** | 10.3% |
| **Rhamnose** | 22.4% |
| **Glucuronic Acid** | 22.5% |
| **Xylose** | 3.7% |
| **Iduronic Acid** | 3.1% |
| **Glucose** | 1% |

### *2.2. Fourier transform infrared spectroscopy (FTIR)*

Extracted polysaccharide powder was analyzed using an IR Prestige-21 apparatus (Shimadzu, Japan). Pre-dried powder was mixed with potassium bromide (KBr), in a mixing ratio of 1:10 of sample:KBr (w/w), and formed into a transparent pellet by manual uniaxial pressing. Transmission spectra were recorded using at least 32 scans with 4 $cm^{-1}$ resolution in the spectral range of 4000 – 400 $cm^{-1}$.

### *2.3. Cell line*

Mouse C3H/An connective tissue fibroblast-like cells (L929) were obtained from the European Collection of Cell Cultures (ECCC, UK). L929 cells between passages 8 and 10 were used to perform the biological studies.

### *2.4. Cell culture*

Cells were cultured in tissue culture flasks at sub-confluent cell density in Dulbecco's modified eagle medium (DMEM) supplemented with sodium bicarbonate (44 mM) (Sigma–Aldrich, Germany), 10% fetal bovine serum (Alfagene, USA) and 1% of antibiotic-antimycotic solution (Gibco, UK). Cultures were maintained at 37°C, in a humidified tissue culture incubator with a 5 % $CO_2$ atmosphere. Medium was renewed every 2-3 days.

### *2.5. Polymer solutions preparation*

Cytotoxicity of extracted ulvan was evaluated according to ISO/EN 10993 part 5 guidelines[28] for an exposure period of 24h. Cytotoxicity assays were performed using latex rubber and standard culture medium as positive and negative controls, respectively. Latex rubber is commonly used as a positive control for standard toxicological assays, as it is known to have a cytotoxic effect inducing cell death and lysis.[35] Hyaluronic acid (HA), was additionally used as a non-cytotoxic reference material.

Ulvan and hyaluronic acid were dissolved in DMEM supplemented with sodium bicarbonate (44 mM), 10% FBS and 1% of antibiotic-antimycotic solution. Fresh stock solutions of ulvan and hyaluronic acid were prepared and diluted to the final test concentrations, in order to obtain polymer solutions with concentrations between 0 and 15 mg/ml.

### *2.6. MTS assay*

The effect on cell viability of exposure to different polymer solutions for a period of 24 h was determined by colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, using CellTiter 96® $AQ_{ueous}$ One Solution Reagent (Promega, USA). Briefly, cells were seeded onto 96-well plates at a concentration of $5x10^3$ cells per well. After 24 h, medium and unattached cells were removed and polymer solutions were added. Culture medium without any polysaccharide was used as negative control and it is represented as 0 mg/ml. Cells were incubated in the presence of the different samples for an additional 24 h period. Three replicates were considered for each sample. After the defined time of exposure, medium was discarded from each well and the cell monolayer was washed twice with sterile phosphate buffered saline (PBS) solution. Cells were exposed to tetrazolium solution (MTS), following manufacturer's protocol, which allowed for viable cells to reduce the tetrazolium compound into a water soluble formazan product. Optical density at 490 nm was measured with a microplate reader (Synergie HT, Bio-Tek, USA).

### *2.7. dsDNA quantification*

As for the MTS test, after cellular confluence the culture medium was replaced by polysaccharide solutions. Three replicates were considered per sample. Culture medium without any polysaccharide was used as negative control (represented as 0 mg/ml). After 24h of incubation media was discarded from each well and the cell

monolayer was washed twice with sterile PBS solution. Cell proliferation was measured by the total amount of double-stranded DNA and quantification was performed using the PicoGreen dsDNA Assay Kit (Invitrogen™, Molecular Probes™, USA), according to the manufacturer's instructions. In brief, cell monolayer was lysed by osmotic and thermal shock and the supernatant used for DNA quantification. Fluorescence was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, in a microplate reader (Synergie HT, Bio-Tek, USA). DNA concentration for each sample was calculated using a standard curve of lambda DNA relating DNA concentration (ranging from 0 to 1.5 µg $ml^{-1}$) and fluorescence intensity.

### *2.8. Total protein quantification*

As for the dsDNA quantification, after reaching confluence the culture medium was replaced by the polysaccharide solutions. Three replicates were considered per sample. Culture medium without any polysaccharide was used as negative control (0 mg/ml). After 24h of incubation media was discarded from each well and the cell monolayer was washed twice with sterile PBS solution. From this point on, Micro BCA™ Protein Assay Kit (Pierce Chemical, USA) was used, following manufacturer instructions. In summary, cells were exposed to a working solution containing bicinchoninic acid (BCA) as detection reagent for $Cu^{+1}$, which is formed when $Cu^{+2}$ is reduced by proteins, producing a purple reaction product. Optical density at 562 nm was measured using a microplate reader (Synergie HT, Bio-Tek, USA). A standard curve was created and sample protein values were read off from the standard graph. Albumin solutions were used as standards (0 to 200 µg/ml). Plotting the measured absorbance against albumin concentration allowed the calculation of protein concentration.

### *2.9. Statistical analysis*

Statistical analysis was performed using the GraphPad Prism statistic software (Release version 5 for Windows). Initial statistical results indicated that nonparametric tests should be used for all comparisons. Therefore, the effect of the test polysaccharides over L929 metabolic activity and cell number was evaluated by Kruskal–Wallis test followed by Dunn's test for multiple comparisons. Direct comparisons between polysaccharides were made by Mann-Whitney test. Statistical significance was defined as $p<0.05$.

## 3. Results

Ulvan is a high molecular weight sulphated polysaccharide composed of rhamnose, glucuronic acid, xylose, iduronic acid and glucose. Ulvan is present in the cell wall of green algae, in close association with proteins and methods of extraction and purification, with a specific deproteinization protocol are not completely effective, as the presence of proteins are still detected.[33-34]

FTIR characterization confirmed the presence of the constituent functional characteristic if this polysaccharide.

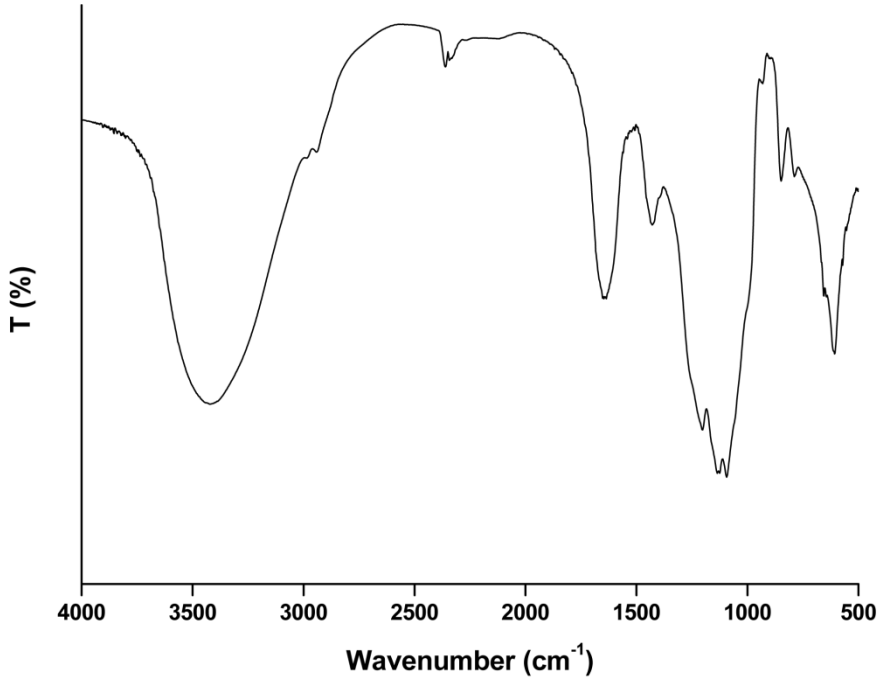


**Figure 4. 1.** Infrared spectrum of ulvan between 400 and 4000 $cm^{-1}$.

Figure 4.1 demonstrates a typical infrared spectrum of ulvan, characterized by signals with different intensities corresponding to 3500, 1650, 1400, 1250 and 1070 $cm^{-1}$.[36-37] Stretching of polymeric hydroxyl groups ubiquitous within ulvan's backbone is identified by a broad band in the range of 3500 – 3200 $cm^{-1}$. Carboxylate groups are revealed by asymmetrical stretching band at 1650 $cm^{-1}$ and symmetric stretching band at 1400 $cm^{-1}$.[33, 37] The region corresponding to 1200 – 1000 $cm^{-1}$ is characteristic of polysaccharides and corresponds to sugar ring vibrations, stretching of C–OH and vibration of the ether glycosidic bridge (CH–O–CH).[37] Stretching of ether sulfate groups ($RO–SO^{-3}$) is represented by a band at about 1250 $cm^{-1}$.[36]

The main objective of the present study is focused on the evaluation of the cytotoxic behaviour of ulvan, reinforced by direct comparison with hyaluronic acid.

In the present research work, cells were cultured in the presence of ulvan, in different concentrations, and subsequently submitted to different assays in order to ascertain on the cytotoxicity of this polysaccharide. Cellular metabolic activity and number were chosen to assess the cytotoxicity of different ulvan solutions with increasing concentrations. Cell metabolic activity is commonly assessed through MTS assay and cell number and proliferation by dsDNA and/or total protein quantification. The result of these assays represents a good indicator of cytotoxicity of a material, as they characterize metabolism and growth of a cell population.

In order to assess and understand ulvan's cytotoxicity, its cytocompatibility was evaluated and compared with hylauronic acid. Taking into account the non-cytotoxic character of hyaluronic acid, this study aimed at comparatively assessing *in vitro* performance of ulvan and authenticating its non-cytotoxic behaviour.

Cellular viability upon exposure to different concentrations of ulvan, evaluated as metabolic activity (Figure 4.2), indicates that this natural origin polysaccharide does not induce a detrimental effect on the metabolic activity of cells. As expected, the same result is observed for hyaluronic acid. However, it appears to be an effect related with concentration for both these polysaccharides. Cells cultured in the presence of different concentrations of these polysaccharides demonstrate equal or higher metabolic activity when compared with negative control (culture medium without the addition of the test sample). In the particular case of ulvan, lower concentrations (0.25, 0.5 and 0.75 mg/ml) induce a significant increase in metabolic activity, which is not observed for higher concentrations (1, 1.25 and 1.5 mg/ml). This increase in metabolic activity of L929 cells exposed to ulvan results in a significant difference on the metabolism of these cells towards the ones exposed to hyaluronic acid, particularly for ulvan concentrations ranging from 0.25 to 1 mg/ml. For hyaluronic acid, this stimulating effect is observed for all test concentrations.

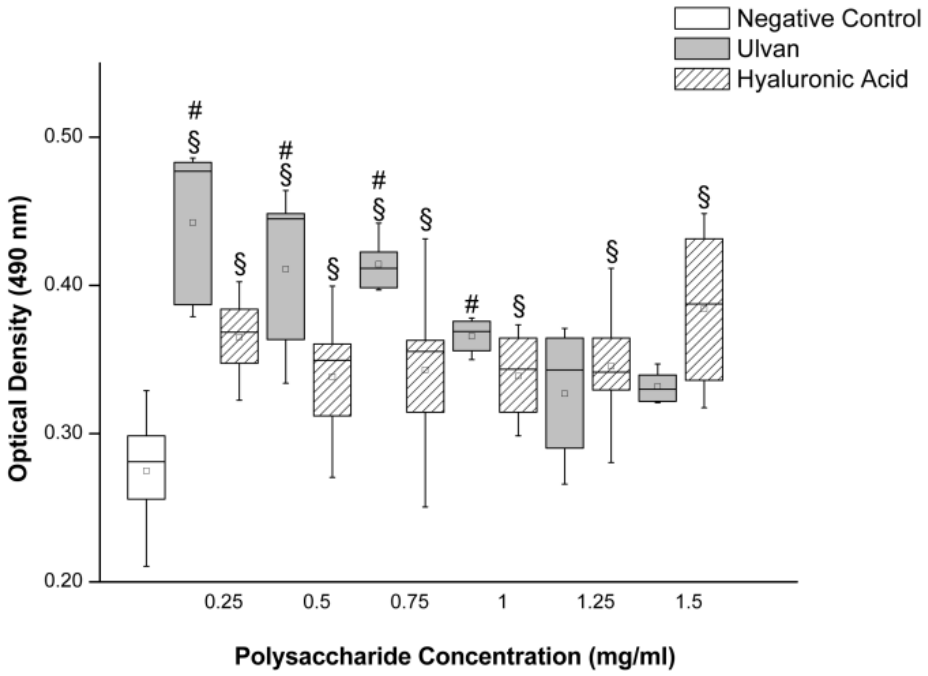


**Figure 4. 2.** Box plot of L929 metabolic activity exposed to two polysaccharides, ulvan and hyaluronic acid. Data were analyzed by nonparametric Mann Whitney and Kruskall Wallis tests: §) denotes significant differences compared with the negative control (0mg/ml) and #) denotes significant differences compared with hyaluronic acid with the same concentration.

Increased metabolic activity observed for cells exposed to ulvan correlates well with the number of cells evaluated through dsDNA quantification (Figure 4.3), which is significantly higher than the one observed in the negative control (for concentrations 0.25, 0.5, 0.75 and 1mg/ml). In the particular case of hyaluronic acid, increased metabolic activity observed in MTS results, is not associated with an increase in the number of cells exposed to different concentrations of this polysaccharide, except for concentration 0.75mg/ml. Some of the differences observed between the metabolic activity of cells exposed to ulvan with the one observed for hyaluronic acid can be correlated with cellular number, which is higher for ulvan than for hyaluronic acid, for 1 and 1.5 mg/l.

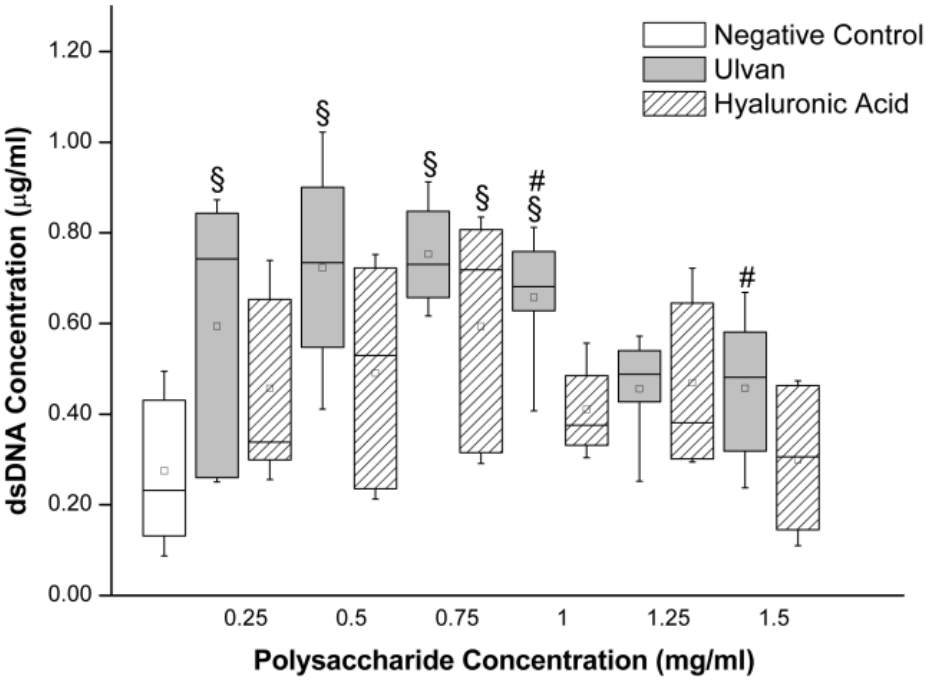


**Figure 4. 3.** Box plot of L929 dsDNA concentration exposed to two polysaccharides, ulvan and hyaluronic acid. Data were analyzed by nonparametric Mann Whitney and Kruskall Wallis tests: §) denotes significant differences compared with the negative control (0mg/ml) and #) denotes significant differences compared with hyaluronic acid with the same concentration.

Despite the differences observed in cellular number, quantified as dsDNA concentration, for various ulvan concentrations in relation to the control, these are not reflected in protein content (Figure 4.4).

Although differences between both polysaccharides relative to protein content are also detected, in this particular case, the majority of the observed significant differences are related with higher protein content in cells exposed to hyaluronic acid (0.75, 1, 1.25 and 1.5 mg/ml).

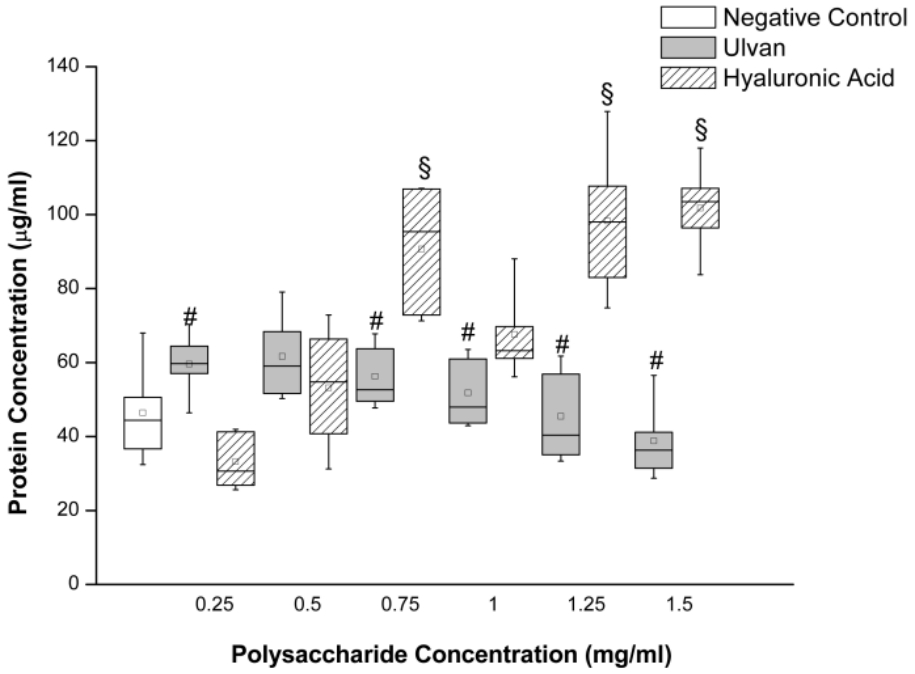


**Figure 4. 4.** Box plot of L929 protein concentration exposed to two polysaccharides, ulvan and hyaluronic acid. Data were analyzed by nonparametric Mann Whitney and Kruskall Wallis tests: §) denotes significant differences compared with the negative control (0mg/ml) and #) denotes significant differences compared with hyaluronic acid with the same concentration.

## 4. Discussion

Nowadays, polysaccharides are the focus of attention in many areas of knowledge. Among these, marine origin polysaccharides are playing a central role, as the marine environment represents an open source of attractive molecules with potential applicability in many fields of interest. Examples of these are chitin and its derivative chitosan, alginate, carrageenan, agar, collagen, among others.[38-43] However, prior to any development towards specific applications, polysaccharides should undergo cytotoxicity screening.

When consumer oriented applications, like food, pharmaceutical and biomedical, are envisaged, evaluation of the cytotoxicity of a polymer assumes a crucial importance, especially when the polymer in focus is a natural extract. In this sense, ulvan cytotoxicity was evaluated and validated by comparison with hyaluronic acid. Applying MTS test, L929 cells produced large amounts of a brown formazan product upon incubation with different concentrations of ulvan, which indicates normal cell metabolism and normal mitochondrial integrity and activity that can be interpreted as a

direct measure of cell viability. In this regard, observed increased metabolic activity induced by low concentrations of ulvan can only mean one of two things – proliferation or an increase in metabolic activity without cell proliferation.[44] In the particular case of ulvan, it is observed an increase in metabolism of L929 cells with significant changes in cellular number (indicated by significant increases on dsDNA concentration), with influence of polysaccharide concentration. In the case of hyaluronic acid, the same observations are valid; only in this case, the majority of differences observed in cell number are detected with protein content. This is to say that cells are metabolic active and viable and are also able to grow in the presence of both polysaccharides. As mentioned before, dsDNA and protein quantification assays can be correlated to the number of cells and provide an indication on cellular proliferation.[45-46] However, in the present study, both assays result in different effects. A possible explanation for these results may lay on the nature of each test; nevertheless both these tests point to a non-cytotoxic behaviour of both polysaccharides, as already observed with MTS results. In this regard, cells appear to retain normal metabolism and growth after exposure to a range of both polysaccharides' concentrations and there appears to be a dose-dependent effect. In fact, a significant increase in the number of cells exposed to some concentrations of ulvan and hyaluronic acid, correlated with an increase in metabolic activity, can be observed by comparison with the negative control. This may indicate that both these polysaccharides may exert an estimulating effect on cellular proliferation. The basis for these observations is beyond the scope of this study; however one could infer about the correlation of this effect with the inherent biological properties associated with each polysaccharide. In the particular case of hyaluronic acid, a stimulatory effect towards cellular proliferation has already been noticed in different studies.[47-48] For example, Ehlers and co-workers found that hyaluronic acid added to culture medium induced a dose-dependent effect on the growth and differentiation of human chondrocytes in culture.[48]

In the present study, an assessment of ulvan's cytotoxicity was performed and results indicate that cellular viability is not impaired by the presence of ulvan, although a dose-dependent effect on cellular metabolism is observed. The same results are observed for hyaluronic acid. In this context, it is possible to infer on ulvan's non-toxic behaviour.

Unravelling the non-cytotoxic nature of ulvan extracted from green algae will favour the exploitation and development of this natural polysaccharide, particularly for consumer oriented applications. The interest in algae origin polysaccharides with novel structures and interesting biological effects for innovative applications is increasing. However, green algae remain largely unexploited in these areas, in comparison with other algae

polysaccharides, including alginate, agar and carrageenan. These are recognized and worldwide accepted polysaccharides for diverse applications, including food and biomedical uses.[1, 6, 42, 49-51] Nevertheless, ulvan is revealing noteworthy potential to be effectively applied in these highly demanding fields.

## 5. Conclusions

Based on data herein reported, ulvan extracted from green algae proved to be cytocompatible, within the test conditions. Our results also reinforce the effectiveness of the applied extraction procedure, yielding a polysaccharide with good biological performance. These findings are further validated by the comparison with hyaluronic acid. Ulvan demonstrates its merit as a promising polysaccharide candidate for biomedical related applications. Data justifies further research on the intrinsic characterization and performance evaluation of this polysaccharide.

## Acknowledgments

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# SECTION IV – PROCESSING OF ULVAN EXTRACTED FROM GREEN ALGAE INTO DIFFERENT STRUCTURES SUITABLE FOR BIOMEDICAL APPLICATIONS

# Chapter 5

# Processing ulvan into 2D structures: cross-linked ulvan membranes as new biomaterials for drug delivery applications

This chapter is based on the following publication: Alves A, Pinho ED, Neves NM, Sousa RA and Reis RL, Processing ulvan into 2D structures: cross-linked ulvan membranes as new biomaterials for drug delivery applications. 2012. International Journal of Pharmaceutics, doi: 10.1016/j.ijpharm.2012.01.021.

## Abstract

The polysaccharide ulvan, composed of sulphated rhamnose, glucoronic and iduronic acids was used to produce polymeric membranes by solvent casting. As ulvan is soluble in water, a cross-linking step was necessary to render the membrane insoluble in water and stable at physiological conditions. Cross-linked ulvan membranes were characterized by Fourier transform infrared spectroscopy, scanning electron microscopy, swelling behaviour was investigated and the mechanical performance assessed by quasi-static tensile testing. Furthermore, the ability and mechanism of sustained release of a model drug from ulvan membranes was investigated. Produced membranes revealed remarkable ability to uptake water (up to ~1800% of its initial dry weight) and increased mechanical performance (1.76MPa) related with cross-linking. On the other hand, medicated ulvan dressings demonstrate the potential as drug delivery devices. Using a model drug we have observed an initial steady release of the drug - of nearly 49% - followed by slower and sustained release up to 14 days. The properties of ulvan membranes herein revealed suggest a great potential of this natural sulphated polysaccharide as a wound dressing.

## 1. Introduction

Wound dressings are widely applied for the treatment of different wounds such as burns, trauma and diabetic ulcers among others.[1-2] Currently commercial available wound dressings can be made of a wide range of materials including polyurethane (Ex: PolyMem®) or gelable polysaccharides, such as starch (Ex: Iodosorb®) and carboxymethylcellulose (Ex: Aquacel®).[1-4] The abundance of available dressings is largely related with the demand of an effective management of the different types of wounds. Furthermore, the complexity of the wound healing process *per se* may require the use of several types of dressings, including medicated dressings.[1] These in particular are meant to provide a sustained delivery of a therapeutic agent for a desired period of time providing aid in the treatment, management (including pain) and eventual healing of a wound.[1]

Different materials are chosen to produce these systems, ranging from natural to purely synthetic polymers. In this context, natural origin materials may offer advantages with respect to biodegradability, biocompatibility and wide availability.[5] Among the diversity of proposed nature origin biomaterials, marine derived polysaccharides are already being investigated for wound dressing applications, namely chitosan and/or alginate.[6-8] In the present study, we have focused our research on ulvan. This rather unexploited polysaccharide is anionic, water soluble, sulphated and semi-crystalline.[9] Green algae, namely *Ulva* the common sea lettuce, are frequently involved in algal proliferation in eutrophicated coastal and lagoon waters.[10] This biomass has diminished associated value and is either employed into compost production or simply dumped.[11] Ways to use and add value to this biomass could be based on specific properties of their cell-wall polysaccharides and their application in high technological areas of knowledge, including the biomedical field. The exploitation of ulvan as an alternative to different synthetic or animal origin polymers would benefit of its algal origin, bioavailability and low expected production costs as well as associated biological activities. In fact, unmodified ulvan possesses interesting properties that can be directed towards highly demanding application areas like the pharmaceutical or food industry.[12-14] However, some degree of modification of polysaccharides is many times required in order to control and fine tune its final properties to particular applications.[15-17] Obtained polysaccharide derivatives may possess desirable properties that can widen their array of applications. In the particular case of ulvan, the study of polysaccharide modification is largely related with the production of biomaterials and reported work in this area relates to functionalization of this polysaccharide.[18] In this pioneer work, Morelli and

Chiellini report the modification of ulvan by grafting with methacryloyl groups in order to produce stable and biodegradable hydrogels aimed for biomedical applications.[18]

Nevertheless, application studies based on ulvan are scarce, especially concerning polysaccharide modification, processing and biomaterial design. Given the peculiar nature of this polysaccharide and its inherent biological effects, it is easy to envisage focused applications based on these properties. Ulvan's biological properties can be related to its sulphation degree, but also with its particular sugar composition.[19-20] For instance, the ubiquitous occurrence of rhamnose in this algal polysaccharide can be considered as an advantageous characteristic, particularly for the treatment of skin pathologies.[21-22] In general, rhamnose-rich polysaccharides demonstrate anti-inflammatory properties, diminish skin bacterial adhesion, protect it from UV-induced and age-related injure and stimulate cellular proliferation and collagen biosynthesis.[21-22] Furthermore, ulvan has been described as a heparinoid agent.[23-25] This heparin-like character positions ulvan as a strategic choice for wound management applications, as heparin, heparin derivatives and heparin-like compounds are known to exert positive effects on chronic wounds.[26] In fact, heparin is often applied in the treatment of wounds, as this sulphated polysaccharide plays a pivotal role in the wound healing process, either by promoting fibroblast proliferation, inhibition of thrombin generation, improvement of fibrinolytic functions, or stimulation of heparin sulphate synthesis.[26-29] Within this context, the sulphated polysaccharide ulvan can be considered for applications in wound management, particularly as a wound dressing. Preparation of polymeric membranes to be applied as wound dressings can be performed by solvent casting, as this is a widespread method to process polymers into membranes.[7-8] Besides ease of preparation and low costs associated with this manufacturing method, it can be an effective way to incorporate a therapeutic agent into the membrane, by mixing it with the polymer prior to casting.[30] After evaporation of the solvent, a combined polymer-drug membrane (medicated dressing) is obtained.[31]

As the fundamental knowledge about ulvan evolves, the development of various modification and processing routes becomes decisive to its progress towards highly demanding areas. Hence the main objective of the present research work is to evaluate the feasibility of ulvan membranes to be applied as a wound dressing, especially as a drug delivery system.

## 2. Experimental procedure

### *2.1. Materials*

Ulvan was extracted from green algae as described elsewhere.[9] 1,4-Butanediol diglycidyl ether (BDDE) and dexamethasone were provided by Sigma–Aldrich (Germany).

### *2.2. Cross-linking ulvan*

Ulvan was mixed with BDDE (1:5 molar ratio), in an alkaline media (sodium hydroxide, 40mM). The cross-linking reaction was allowed to proceed for 180min, at 50ºC. Cross-linked ulvan powder was exhaustively washed with water and acetone to remove any residual cross-linker. The obtained dried powder was further used to produce cross-linked ulvan membranes.

### *2.3. Membrane preparation*

To prepare the polymeric membranes, cross-linked ulvan was dispersed in water (1% w/v) and the solution was homogenised with an UltraTurrax apparatus. Ulvan membranes were prepared by casting ulvan solution on Petri dishes, followed by solvent evaporation, at 50ºC, in a vacuum oven.

Ulvan membranes impregnated with dexamethasone were prepared as described above, except that dexamethasone was dissolved in acetone and added to ulvan solution, with a final concentration of 15% w/w, prior to solvent casting. In the present study, dexamethasone, a steroid anti-inflammatory drug, was used as a model therapeutic agent. Non cross-linked ulvan membranes were prepared as described to study the cross-linking mechanism and the influence of cross-linking on the overall mechanical performance. The final appearance of ulvan membranes, loaded or not with dexamethasone, is a homogeneous and transparent yellowish film.

### *2.4. Fourier transform infrared spectroscopy (FTIR)*

Chemical modifications introduced by the cross-linking reaction were investigated by FTIR. The infrared spectra were recorded on a spectrophotometer (IR-Prestige-21, Shimadzu, Japan), controlled by IRsolution software. All spectra were averaged on 32 scans in the range of 600–4400 $cm^{-1}$ with a resolution of 4 $cm^{-1}$.

### *2.5. Scanning electron microscopy (SEM)*

Surface morphology of the produced membranes was analyzed using a Leica Cambridge S-360 scanning electron microscope (SEM) (Leica Cambridge, England). All specimens were pre-coated with a conductive layer of sputtered gold.

### *2.6. Mechanical properties testing*

Ulvan membranes were subjected to tensile tests to evaluate the effect of cross-linking over the mechanical properties and to determine the mechanical performance of these membranes. Membranes with 500µm in thickness were cut into 10-mm-long specimens and tensile tests, in dry state, were performed using a Universal Testing Machine (Instron 4505, UK), with a load cell of 1 kN, gauge length of 10 mm and cross-head speed of 5mm/min was used up to rupture of the membrane. A minimum of five specimens were tested for each sample (the values reported are the average of those results). These tests allowed the determination of different mechanical properties defined as follows: tensile modulus defined as the slope of the straight line obtained by linear regression of the stress-strain curve in the near elastic region of the material (between 0 and 1.0 % strain); ultimate tensile strength (referred as tensile strength), defined as the maximum tensile stress developed in the material during the tensile test and strain at break point (referred as tensile strain), defined as the maximum strain of the material, i.e. elongation at the failure point of the material.

### *2.7. Water uptake study*

The hydration degree of ulvan polymeric membranes was assessed over a period of 14 days. Five specimens (previously weighed) were immersed in phosphate buffered saline (PBS, pH 7.4) and incubated at 37°C for 1, 3, 7 and 14 days. After each defined period of time, the specimens were removed from the PBS solution, gently blotted with a paper filter and the weight was again measured. The water uptake was calculated by the following equation:

$$Water\ Uptake(\%) = [(w_s - w_i) \div w_i] \times 100$$

where $w_i$ is the initial weight of the specimen before immersion and $w_s$ is the wet mass of the specimen at time t (days) after being removed from the solution.

### *2.8. In vitro dexamethasone release profile*

Five specimens of ulvan cross-linked membranes loaded with dexamethasone were immersed in PBS (pH 7.4) and incubated at 37°C for a period of 14 days. The release of dexamethasone was periodically monitored by extracting 500µl aliquots and

replenishing with 500µl of PBS, in predetermined time intervals. The concentration of dexamethasone was determined by UV–Vis spectroscopy at 242nm (Shimadzu UV 1601, Japan). The results presented are an average of five measurements.

### *2.9. Mathematical modeling of dexamethasone release from ulvan membranes*

To better understand the mechanisms of dexamethasone release from ulvan membranes the power law (or Peppas equation) was applied to the experimental data.[32-33] This particular model was chosen as it accounts for both drug diffusion and matrix swelling. Power law is a simple empirical equation, which describes a linear relationship between logarithm of the amount of drug released and logarithm of time, up to 60% of the maximum drug released:

$$\frac{M_t}{M_\infty} = kt^n$$

$M_t/M_\infty$ is related with the fractional drug release, $k$ is the kinetic constant, $t$ refers to the release time and $n$ is the diffusional exponent that is related to the drug transport mechanism. For a thin polymeric membrane, the drug release mechanism is Fickian diffusion when $n$=0.5, $n$=1 Case II transport occurs leading to zero-order release and if the value of $n$ is between 0.5 and 1, anomalous transport is observed.[32-34]

## 3. Results and Discussion

In the present research work, the development of cross-linked ulvan membranes is described and their use for local delivery of drugs, as medicated dressings, is proposed. In this type of applications, stability in physiological conditions constitutes a desired property. As unmodified ulvan is readily dissolved in water cross-linking plays a decisive role in tuning the properties of the final polymeric matrix.

BDDE is one of the most frequently used homobifunctional epoxide reagent to cross-link polysaccharides. It is considered cytocompatible which is of extreme importance when we are focusing on biomedical applications.[35] The epoxide functionalities of BDDE are able to react with different groups like hydroxyls, amines or thiol groups.[36-37] Polyexpoxide compounds have already been successfully used to cross-link collagen, gelatine and hyaluronic acid.[35-36] In fact BDDE is the cross-linking agent used to cross-link many commercially available products, namely in aesthetics, such as dermal fillers like Juvederm® or Restylane®.[38]

For all the above reasons, chemical modification of ulvan was performed via cross-linking with BDDE. In order to try to identify the bonds responsible for the cross-linking

of ulvan with BDDE and understand the mechanism of reaction, FTIR spectroscopy was performed on the processed membranes, with and without cross-linking. By analyzing the FTIR spectra of Figure 5.1, it is possible to identify different characteristic peaks of ulvan: stretching (st) vibration of polymeric hydroxyl groups at 3500-3200cm$^{-1}$; it is also possible to detect the presence of carboxyl groups by a band at 1650cm$^{-1}$, the asymmetric stretching of the ether glycoside bridge and the symmetric and asymmetric stretching of the ether sulphate group in the range of 1315–1220cm$^{-1}$ and 1140–1050cm$^{-1}$.[9] By comparing FTIR results before and after cross-linking, it is not possible to detect the formation of novel bonds, which could have been introduced during the cross-linking reaction. There are several groups present in the region of 900–1250cm$^{-1}$ (highlighted in Figure 5.1), characteristic of native ulvan. However, after cross-linking, the signal around 1090cm$^{-1}$ is markedly decreased. The disappearance of this signal may be due to the consumption of –OH needed for the cross-linking reaction to occur, as this band can be attributed to CH – OH (st).

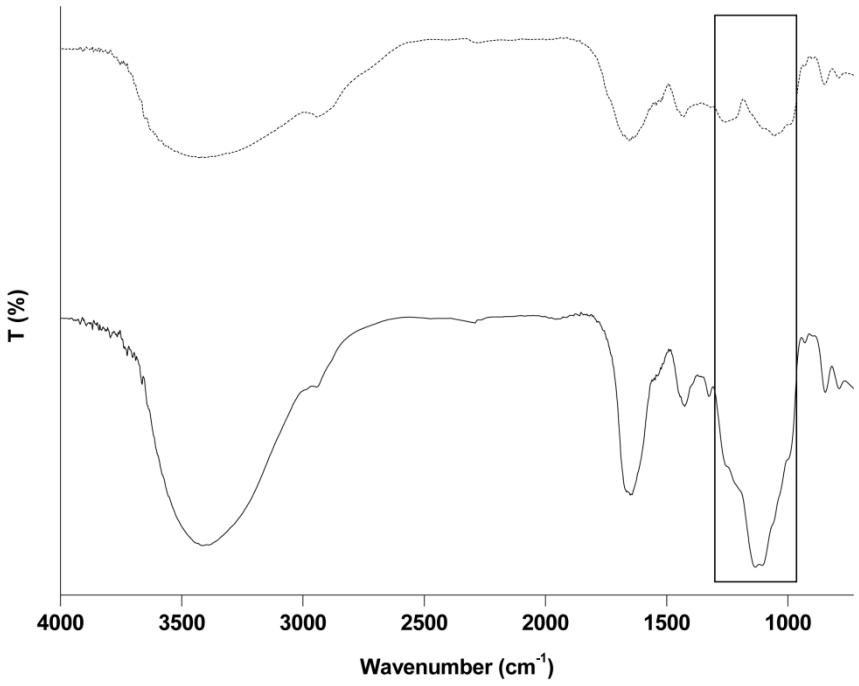



**Figure 5. 1.** FTIR spectra (Straight line: ulvan; Dash line: cross-linked ulvan).

Epoxide groups are able to react with different nucleophiles, including amines, thyols and hydroxyl groups, present within the polymer backbone, yielding stable covalent bonds.[37, 39-40] Reaction with different nucleophyles, in appropriate experimental conditions, will result in different end bonds. For example, efficient coupling of epoxide moieties with hydroxyls will occur in an alkaline media, at high pH, yielding stable ether

bonds; primary amine groups react with epoxide groups at moderate alkaline pH, resulting in secondary amines or sulphydryl groups yield thioether bonds in a reaction with epoxides at close to neutral pH.[39] In this scheme, given that reaction of ulvan with BDDE occurs in an alkaline moiety, at high pH, and considering the apparent consumption of hydroxyl groups, as demonstrated by FTIR, ether bonds are expected to be formed.

A reaction mechanism is proposed and illustrated in Figure 5.2. Reaction is thought to occur via ulvan's hydroxyl groups that are able to react with the epoxide moiety of BDDE in alkaline media, yielding a stable ether bond. Cross-linking may be complete, bringing together two polysaccharidic chains via ether bonds, or incomplete, with epoxide pendant groups.[36] Given the bifunctional nature of BDDE, pendant epoxide groups may be expected, due to limited reagent concentration or due to absence of vicinal functional groups available to react with the epoxide.[36]

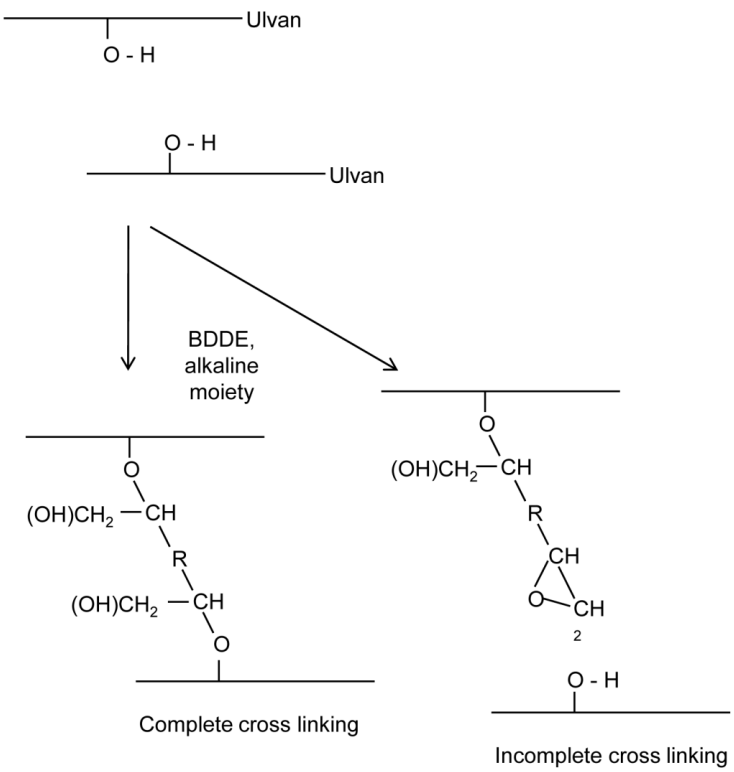
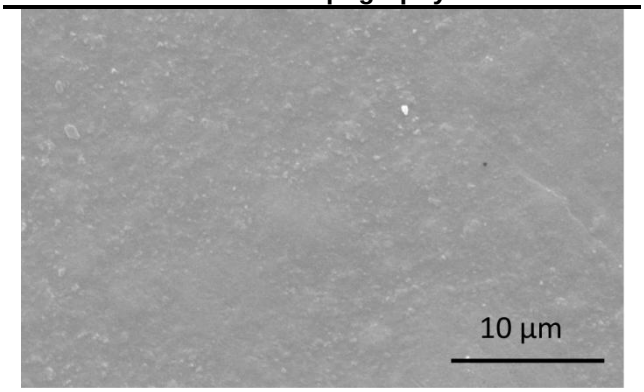



**Figure 5. 2.** Proposed cross-linking mechanism of ulvan polysaccharide with BDDE.

Surface studies by SEM revealed that ulvan membranes are non porous and their surface is homogeneous, presenting some degree of roughness (Table 5.1).

**Table 5. 1.** Surface topography of produced ulvan membranes.

| Surface topography |
| --- |
|   SEM micrograph of the surface of cross-linked ulvan membrane (Magnification 3000x) |

As ulvan membranes are envisaged for applications as dressings, they are required to be resilient and stress resistant to be able to cope with the stresses exerted during manual manipulation or by surrounding tissue, and this endorses the importance of mechanical behaviour on this particular applications.[1] To better understand the influence of cross-linking on the mechanical performance of the designed membranes, a comparison between non cross-linked membranes and cross-linked membranes was performed. When comparing the results obtained for both types of ulvan membranes, it is notorious the striking effect of cross-linking on the mechanical performance of ulvan membranes; in general, these properties can be improved by cross-linking.[41-42] Tensile strength and modulus were significantly improved by the cross-linking reaction (Table 5.2). Membranes without cross-linking have a tensile strength of 4.7kPa and a tensile modulus of 580kPa. Cross-linking ulvan yielded membranes with increased tensile strength and tensile modulus, causing the tensile strength to increase to 44kPa and the tensile modulus to increase by 203%. However, this made ulvan membranes less ductile, as it can be seen by the decrease in tensile strain.

**Table 5. 2.** The mechanical properties obtained from the tensile curves are presented in the following table.

| Ulvan Membrane | Tensile Modulus (kPa) | Tensile Strength (kPa) | Tensile Strain (%) |
| --- | --- | --- | --- |
| **Without cross-linking** | 580.0 ± 213.00 | 4.7 ± 1.89 | 26.4 ± 10.01 |
| **With cross-linking** | 1760.0 ± 284.00 | 44.0 ± 12.00 | 15.2 ± 7.66 |

In order to evaluate the swelling behavior of ulvan membranes they were placed in a buffer solution, at 37ºC. Figure 5.3 presents the profile of the water-uptake capability of cross-linked ulvan membranes as a function of time and demonstrates that ulvan membranes possess significant water uptake ability, showing a maximum peak after 14 days in PBS (~1800%). The ability to uptake water must be one of the most remarkable and defining properties of the produced ulvan membranes. Maintenance of moisture in a wound is considered a critical aspect of wound management as a moist environment may favor the healing process through prevention of desiccation and cell death, stimulation of cell migration, angiogenesis, connective tissue generation and autolysis.[1, 43] Furthermore, if high water absorption from exudate wounds is desired, ulvan membranes' hydration ability assumes a pivotal role.[1] In this context, the water uptake demonstrated by ulvan membranes becomes relevant and supports their feasibility to be applied as wound dressings.

On the other hand, this knowledge becomes relevant particularly when these membranes are aimed for drug delivery applications.

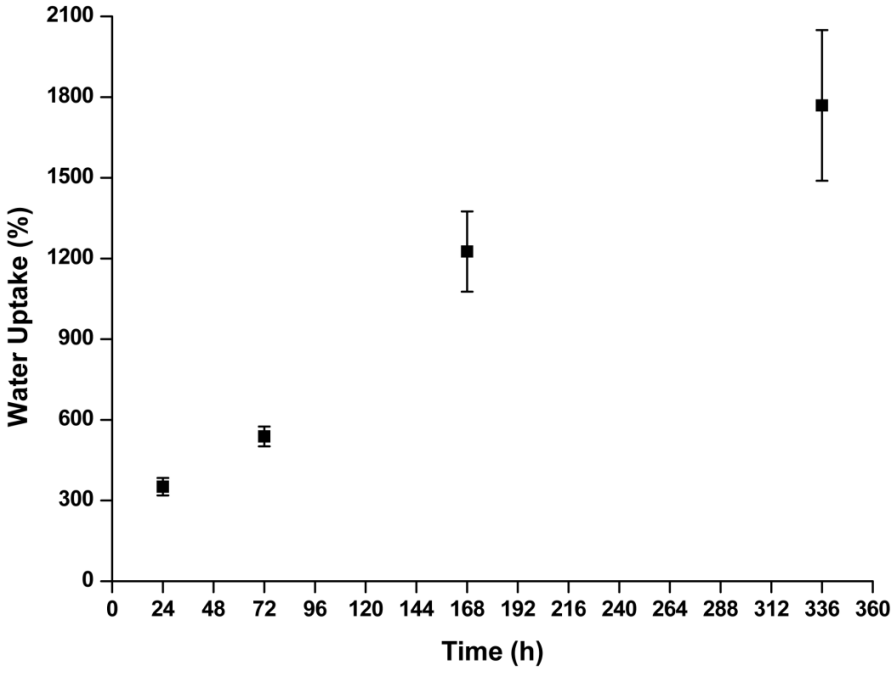



**Figure 5. 3.** Percentage of water uptake by cross-linked ulvan membranes along time.

In the present study, cross-linked ulvan membranes were loaded with a therapeutic model drug, dexamethasone, so as to evaluate their ability to be further used as medicated wound dressings. From Figures 5.4, and in more detail in Figure 5.5, a steady release of dexamethasone is observed for 8 hours. At this point, 49% of drug has been release from the prepared membranes. Afterwards, a slower release of the

drug is detected and at day 14 around 72% of dexamethasone has been released from ulvan membranes.

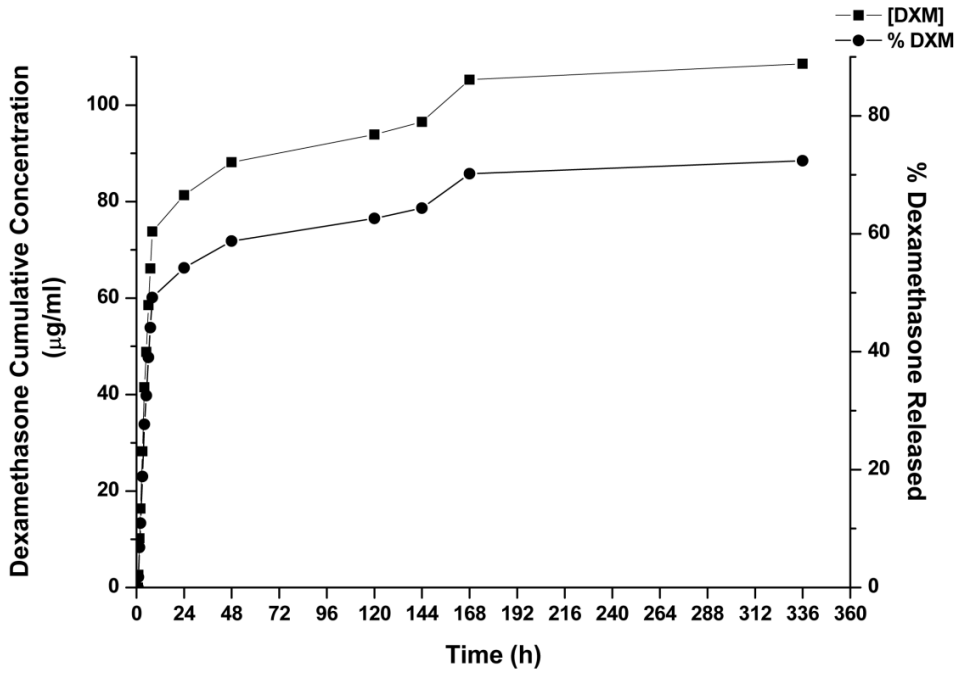



**Figure 5. 4.** Dexamethasone concentration and percentage of drug released from ulvan membranes (SD for *n*=5).

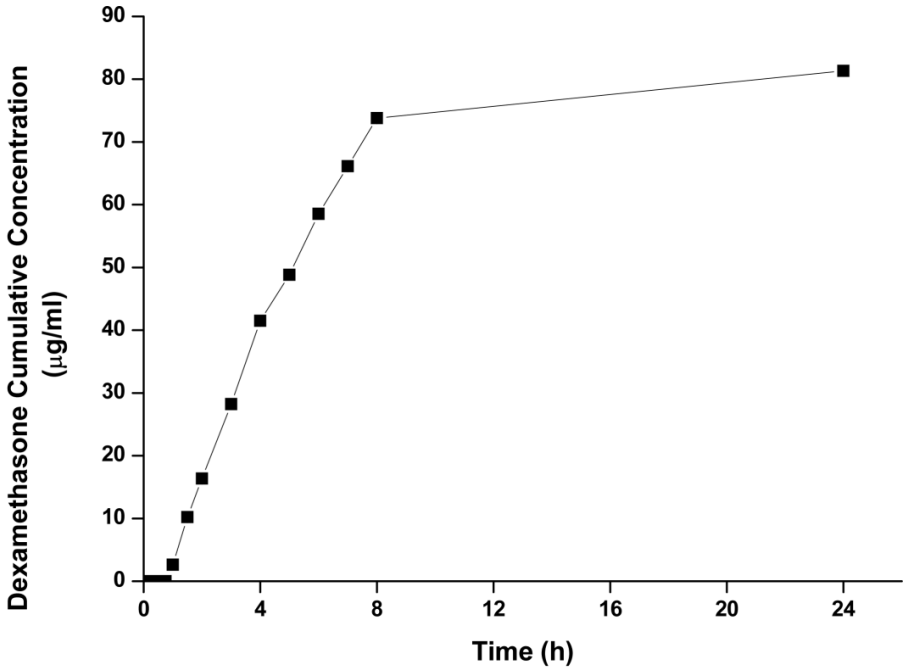



**Figure 5. 5.** Detail of dexamethasone concentration release profile from ulvan membranes (SD for *n*=5).

There are several factors that influence the release of a drug from a matrix, including diffusion of the drug out of the matrix and the water uptake by the polymeric matrix.[34] One factor is mostly related with the drug itself and the other is related with the polymer and the polymer network. Given the water uptake ability of ulvan membranes, we can hypothesize that the influence of ulvan's swelling on the release of the drug is significant. As ulvan membranes are regarded as a non-porous system, drug molecules are released through the network meshes within the polymeric phase.[30] This theoretical scenario is supported by the mathematical modelling of the release of dexamethasone from the prepared ulvan membranes. Fitting the power law equation to the obtained data, kinetic parameters that describe the mechanism of release associated to this membrane system were determined. This is a simple semi-empirical mathematical model describing a linear relationship between the logarithm of the amount of drug released and logarithm of time, up to 60% of the maximum drug released.[32-34]

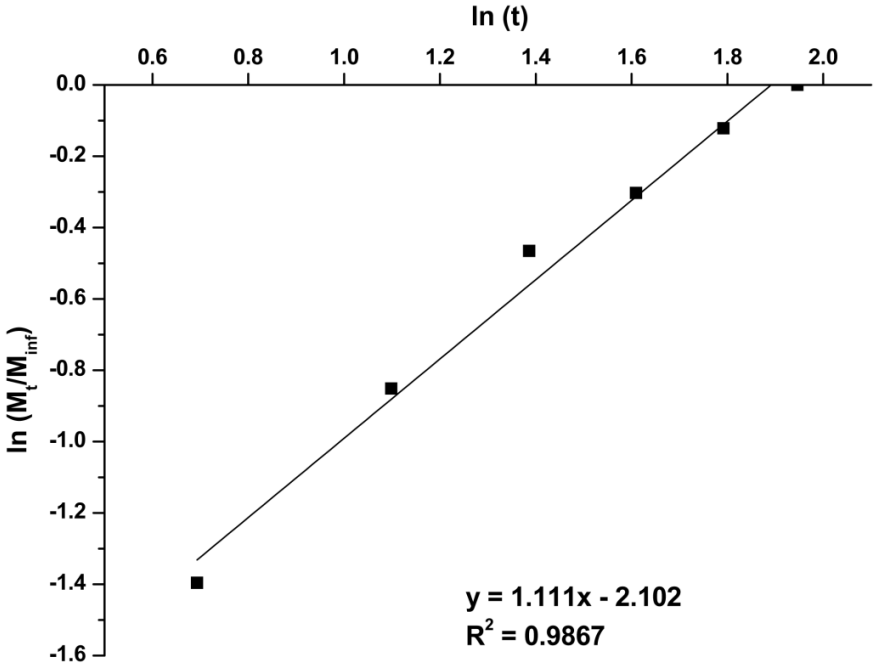



**Figure 5. 6.** Power law applied to the drug release profile of ulvan membranes loaded with dexamethasone.

From the slope of the plot of logarithm of $M_t/M_\infty$ versus logarithm of time, $n$ (the exponent characterizing the release process) was calculated (Figure 5.6). In the particular case of ulvan membranes, $n$ is close to 1, which indicates that release of dexamethasone follows a non-Fickian release mechanism, specifically Case-II transport, also described as zero-order kinetic.[32] In this particular case, the release of

dexamethasone from ulvan membranes is associated with the relaxation of the polysaccharide upon hydration.

## 4. Conclusions

Ulvan membranes meant to be used as wound dressings or as vehicles to deliver therapeutic agents in the context of wound management have been proposed. As part of the design of these membranes, successful chemical cross-linking of ulvan was achieved with the epoxide BDDE, via the formation of ether bonds through ulvan's hydroxyl groups. The properties of ulvan membranes herein presented suggest that this natural sulphated polysaccharide can be proposed for the envisaged applications as wound dressings. Furthermore, the release of dexamethasone from ulvan membranes proceeds in a sustained fashion, which supports the feasibility of these membranes to be used as drug delivery systems as medicated wound dressings. As the application development of this polysaccharide is in its early stages, the herein described ulvan processing techniques and envisaged applications pose an innovative attempt to add value to this rather unexploited algae derived polysaccharide.

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# Chapter 6

# Processing of degradable ulvan 3D porous structures for biomedical applications

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This chapter is based on the following publication: Alves A, Sousa RA and Reis RL, Processing of degradable ulvan 3D porous structures for biomedical applications. 2012. Submitted.

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## Abstract

The interest in ulvan within a biomedical framework increases as the knowledge of this polysaccharide evolves. Ulvan has been recently proposed as a potential biomaterial, and scaffolds based on this polysaccharide are now being studied for different tissue applications. In this work, a novel porous structure based on cross-linked ulvan was designed and characterized. Its mechanical performance and water uptake ability were assessed, morphology analysed through scanning electron microscopy and morphometric parameters quantified by micro-computed tomography. Cell viability and cell proliferation were evaluated in order to estimate the cytotoxicity of these structures, and respective degradation products. Produced ulvan structures revealed remarkable ability to uptake water (up to ~2000% of its initial dry weight) and are characterized by a highly porous and interconnected structure. Furthermore, these ulvan structures underwent non-toxic degradation and cells remained viable through the time of culture. These results position ulvan structures as prospective blocks that can be further functionalized in order to acquire the desired and needed biological interactivity to be used as tissue engineered structures.

## 1. Introduction

Scaffold design is a key aspect of tissue engineering. Scaffolds are intended to support cell colonization and neo-tissue formation, mimetizing the tissue extracellular matrix.[1-2] In general, a scaffold should be biocompatible, biodegradable, with suitable morphological properties and mechanical performance.[1, 3] Ideally, it should not induce cytotoxicity and should interact with cells encouraging cellular functions such as adhesion, growth and migration. It should degrade in a non-toxic manner as the neo-tissue forms and possess adequate mechanical integrity to withstand biological forces and maintain physical integrity. Finally, it should have adequate porosity and surface area in order to ensure cell colonization, survival and support neo-tissue formation.[3-4]

Nowadays, polysaccharides are considered, for many reasons, an attractive option for the design of a scaffold to repair or regenerate a tissue. First of all, polysaccharides are, in general, non-cytotoxic, with different and noteworthy biological activities.[3, 5-7] Secondly, polysaccharides represent a key component of the extracellular matrix, and many times determine its properties and functionality.[4] Overall, the chosen material should be able to mimic, as close as possible, the components and properties of the tissue that is going to be repaired, regenerated or substituted.[7] Polysaccharides and/or proteins naturally present in the human body tend to be a recurrent choice, namely hyaluronic acid, chondroitin sulphate, collagen or fibrin.[3, 8-12] Polysaccharides that present similar traits to these mammalian glycosaminoglycans are also frequently used as biomaterials.[8, 10, 13-14]

In this context, ulvan remains a rather unexploited green algae polysaccharide. This polysaccharide is mainly composed of uronic acids and sulphated rhamnose, which represent the basis of its principal constituent unit – ulvanobiuronic acid.[15-18] Ulvanobiuronic acid A is composed of glucuronic acid and rhamnose and ulvanobiuronic acid B is composed of iduronic acid and rhamnose.[16] The presence of both rhamnose and iduronic acid confers a particularly peculiar character to this polysaccharide, as both this monosaccharides are rarely found in algal polysaccharides.[17-18] However, it is the presence of both iduronic acid and a sulphated sugar moiety that is the basis of the comparison of ulvan to mammalian sulphated glycosaminoglycans, especially sulphated chondroitin or dermatan.[15, 18] Given this similarity, it is easy to envisage its potential use in areas like the biomedical field. In this regard, it is herein proposed a processing method to obtain a three dimensional porous structure, based on ulvan, to be applied as a medical device, particularly for tissue engineering applications. In order to render these structures suitable for biomedical applications, a chemical modification step was introduced in order to confer stability in

physiological conditions, control water uptake ability and provide improved mechanical properties. Both carboxyl and hydroxyl groups of ulvan are attractive sites for chemical modification and it may be achieved with different commercially available cross-linking agents. In the present work, we have chosen to work with 1,4- butanediol diglycidyl ether (BDDE) due to its acceptability and applicability in biomedical, pharmaceutical or cosmetic applications.[19-22] As a practical example, this agent is widely used to cross-link hyaluronic acid to produce commercially available stable fillers for dermatological purposes.[19]

In summary, the main objective of the herein described research work is to produce a 3D porous structure based on cross-linked ulvan, with defined physicochemical properties and evaluate its feasibility to be applied in a tissue engineering context.

## 2. Materials and methods

### *2.1. Materials*

Ulvan was extracted from green algae, namely *Ulva lactuca*, using a procedure described elsewhere.[23] Briefly, Soxhlet extraction of dried *Ulva lactuca* removed most of the lipids and colouring matter. The residual off-white weed was dried and subjected to three hot-water extractions, between 75-85ºC. After filtration through a cotton cloth, aqueous extracts were centrifuged, and the liquid supernatant was filtered. The water extract was concentrated until 10-20% of its initial value, in a rotary evaporator. Starch and proteins were removed by enzymatic digestion. Afterwards, the solution was decolorized and deodorized by adsorption on activated charcoal. The water extract was centrifuged, filtered and precipitated with 4 vol. of absolute ethanol. Finally, the recovered precipitate was freeze dried. Yield of a white polysaccharide resulting from this extraction methodology is c.a. 10 – 20%.

Unless otherwise specified, all chemicals were bought from Sigma–Aldrich (Germany) and used as received.

### *2.2. Synthesis of chemically cross-linked ulvan porous structures*

Ulvan was chemically cross-linked with 1,4 – butanediol diglycidyl ether (BDDE) by mixing the polysaccharide with the cross-linking agent in an alkaline medium, to favour the formation of stable ether bonds. The reaction was allowed to proceed for 180 minutes, at 50ºC. Afterwards, the solution was dialyzed against distilled water to remove any unreacted cross-linker, placed in an appropriate mould and freeze dried to allow the formation of porous samples (condenser temperature: ~80ºC, vacuum:

0.030mbar, for a minimum of 3 days). pH of the final ulvan solution prior to freeze drying was controlled (pH~6). Ulvan and cross-linker concentrations are reported as a weight ratio between cross-linker (referred as XL) and ulvan (referred as UL) and are presented in Table 6.1.

**Table 6. 1.** Different formulations based on ulvan (UL) and cross-linker (XL) used to prepare various ulvan porous samples.

| [UL] (% w/v) | XL (weight ratio) |
|---|---|
| **4** | 0.20; 0.27; 0.53 |
| **5** | 0.14; 0.50; 1.00; 1.50; 2.00 |
| **6** | 0.13; 0.18; 0.36; 0.57 |
| **8** | 0.25; 0.50; 0.75; 2.00 |

### *2.3. Fourier transform infrared spectroscopy (FTIR)*

Chemical modifications introduced by the cross-linking reaction were investigated by Fourier transform infrared spectroscopy (FTIR). Infrared spectra were recorded on a spectrophotometer (IR-Prestige-21, Shimadzu, Japan), controlled by IRsolution software. All spectra were averaged from 32 scans in the range of 600–4400cm$^{-1}$ with a resolution of 4cm$^{-1}$.

### *2.4. Mechanical compression tests*

Samples were mechanically tested by compression experiments in an Instron 5543 (Instron Int. Ltd., UK) universal testing machine with a load cell of 1kN. Compression testing was carried out at a crosshead speed of 2mm/min, until obtaining a maximum reduction in samples' height of 60%. Compressive modulus is defined as the slope of the straight line obtained by linear regression of the stress-strain curve in the near elastic region of the material (between 0 and 1.0 % strain). A minimum of five samples of each type/condition were tested.

### *2.5. Water uptake and degradation tests*

Samples were previously weighted and placed into screw-top plastic tubes. These were immersed by adding 5mL phosphate buffered saline (PBS) and allowed to swell at 37ºC under agitation (60rpm). After each time point (1, 3, 7, 14 and 21 days), samples were placed on a Whatman filter for 10s in order to remove non absorved water, and subsequently weighted.

Water content in the swollen scaffolds was calculated by the following equation:

$$Water\ Uptake\ (\%) = [(Ws - Wi)/Wi] \times 100$$

where $w_i$ is the initial weight of the specimen before immersion and $w_s$ is the weight of the swollen structure. A minimum of three samples for each type/condition were analysed.

After each time period, the samples were dried and weighted to determine the weight loss, which was calculated according to the equation:

$$Weight\ Loss\ (\%) = [(Wd - Wi)/Wi] \times 100$$

where $w_d$ is the final weight of the sample (dried after immersion) and $w_i$ is the initial weight of the sample. Presented data is the result of the average of at least three measurements.

### *2.6. Scanning electron microscopy (SEM)*

Produced samples were analyzed using a FEI Nova 200 (FEI, USA) scanning electron microscope (SEM). All specimens were pre-coated with a conductive layer of sputtered gold.

### *2.7. Micro-computed tomography (μ-CT)*

Samples herein described were analyzed by micro-computed tomography (μ-CT) using a high-resolution micro-CT SkyScan 1072 scanner (Skyscan, Belgium) in order to characterize its morphological and morphometric properties. The X-ray source was set at 32kV of energy and 191μA of current, and resolution of pixel size of 6.69μm and exposure time of 1.8sec. A minimum of three samples of each type/condition were analysed. After reconstruction (NRecon, SkyScan, Belgium) of the acquired data, bitmap of 1024x1024 pixels were obtained and representative binary images of approximately 100 slices with a dynamic threshold of 61–255 (grey values) were generated and analyzed (CT Analyser®, SkyScan, Belgium). Different morphometric parameters were determined, including porosity, mean pore size and respective distribution and scaffold interconnectivity. This last parameter was calculated assuming two minimum pore sizes, of 13.4μm and 53.5μm, that is to say that interconnection diameter lower than these values were not considered as open pores. In this sense, interconnectivity of the scaffold is calculated according to the formula:

$$I = \left[\left(V_{totalpore} - V_{disconnectedpore}\right) / V_{totalpore}\right] \times 100$$

where the volume of the disconnected pore stands for the disconnected pore volume.

### *2.8. Cell viability and proliferation*

Confluent L929 cell at passages 20–26 were harvested for seeding onto ulvan samples at a density of $5.0 \times 10^5$ cells/scaffold.[24] The constructs were cultured in standard

medium for 1, 3, and 7 days. Cells seeded on standard tissue culture polystyrene were used as a negative control, representing cell normal growth in culture media. In this experiment cell adherent culture plates were chosen; cells non-adherent to the samples were used, as a non standard method to screen possible toxicity of soluble components and other degradation products resulting from ulvan samples.

Cell viability for each culturing time was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, USA), following manufacturer instructions. Absorbance was measured at 490 nm for a minimum of four samples per condition and time point, using a microplate reader (Synergie HT, Bio-Tek, USA). Cell proliferation was quantified by the total amount of double-stranded DNA (dsDNA), along culturing time. Quantification was performed using PicoGreen dsDNA Assay Kit (Invitrogen™, Molecular Probes™, USA), according to the manufacturer's instructions. Briefly, cells were lysed by osmotic and thermal shock and the supernatant used for the DNA quantification assay. Fluorescence was measured at an excitation wavelength of 485/20nm and at an emission wavelength of 528/20nm, in a microplate reader (Synergie HT, Bio-Tek, USA). Quadruplicates were made for each sample and per culturing time. dsDNA concentration for each sample was calculated using a standard curve relating DNA concentration (ranging from 0.0 to 1.5mg/mL) and fluorescence intensity. Cell viability and dsDNA quantification were performed for cells adherent and non-adherent to the produced samples.

### *2.9. Cell Imaging*

Viability and morphology of cells on ulvan samples were assessed after 1 and 3 days, by reflected/transmitted light microscopy, following calcein-AM and propidium iodide staining, and by scanning electron microscopy (SEM). For reflected/transmitted light microscope visualization, ulvan samples were incubated in culture medium with calcein-AM and propidium iodide (Invitrogen™, Molecular Probes™, USA) for 30min. Calcein-AM is a non-fluorescent permeable compound that once inside viable cells is converted by intracellular esterases into a fluorescent cell impermeable form, being used as a green fluorescent indicator of viable cells. Propidium iodide is used as a red fluorescent marker of cells with compromised membranes, being used as an indicator of dead cells. The calcein-AM/propidium iodide stained samples were placed on a microscope slide and observed by reflected/transmitted light microscope (Zeiss, Axio Imager.Z1m, Germany). For SEM analysis, ulvan samples were fixed with 2.5% glutaraldehyde, in PBS, for 1h at 4ºC. Samples were further dehydrated in increasing ethanol concentrations, critical point dried and gold sputter coated prior to SEM observation.

### *2.10. Statistical analysis*

Statistical analysis was performed using GraphPad Prism statistic software (Release version 5 for Windows). All sample results were previously tested for normal distribution using Shapiro–Wilk test. Negative results indicated the adoption of nonparametric tests for all comparisons. Therefore, the effect of the produced structures over L929 viability and proliferation over time was evaluated by Mann-Whitney test and Kruskal–Wallis test followed by Dunn's test for multiple comparisons. Direct comparisons of both produced scaffolds were made by Mann-Whitney test. Statistical significance was defined as $p<0.05$.

## 3. Results

In order to confirm the expected mechanism of cross-linking reaction of ulvan with BDDE, FTIR spectroscopy was performed on ulvan structures, with and without chemical modification.

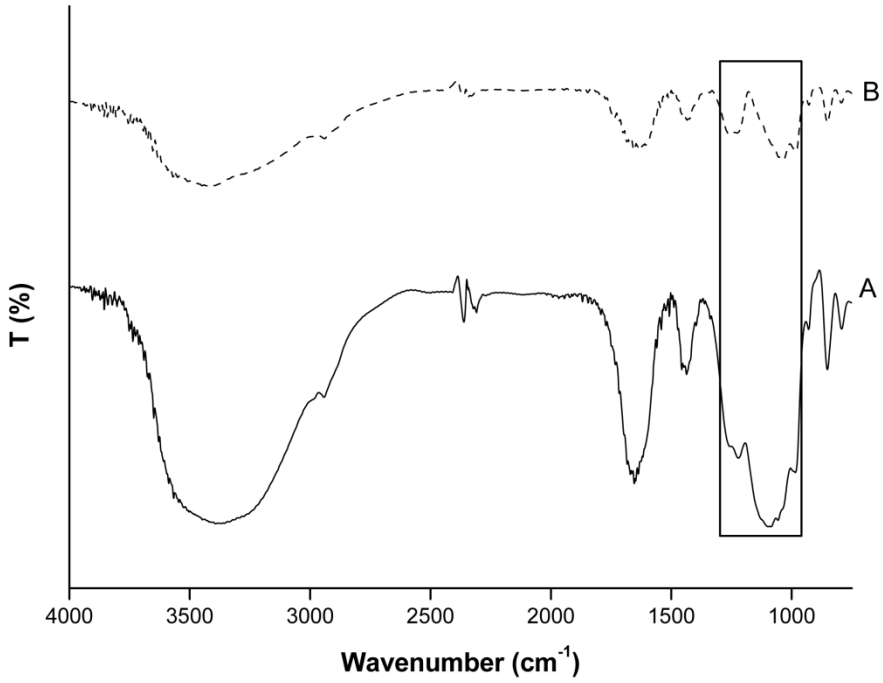


**Figure 6. 1.** Infrared spectra of ulvan structures between 600 and 4000cm$^{-1}$. A: structure without cross-linking; B: cross-linked structure. The main difference between both spectra is highlighted.

By means of analyzing FTIR spectra of Figure 6.1, it is possible to identify different characteristic bands of ulvan: stretching (st) vibration of polymeric hydroxyl groups at 3500-3200cm$^{-1}$; it is also possible to detect the presence of carboxyl groups by a band

at 1650cm$^{-1}$, the asymmetric stretching of the ether glycoside bridge and the symmetric and asymmetric stretching of the ether sulphate group in the range of 1315–1220cm$^{-1}$ and 1140–1050cm$^{-1}$.[23] The signal detected around 1090 cm$^{-1}$ present in native ulvan structures, without chemical modification, is not detected after cross-linking and may be due to the use of –OH groups needed for the cross-linking reaction to occur, as this band can be attributed to CH–OH (st). Cross-linking of ulvan with BDDE has already been successfully performed to produce ulvan membranes and the proposed mechanism of reaction is suggested to involve hydroxyl groups present within the backbone of the polysaccharide and epoxide groups of the cross-linker.[25]

Different ulvan structures were produced with varying concentrations of polysaccharide and cross-linker and characterized and evaluated by different criteria, including mechanical performance, water uptake and degradation time represented as structural stability over time in PBS. These results are summarized in Table 6.2.

**Table 6. 2.** Mechanical, hydration and stability properties of different ulvan structures produced by varying the concentration of polysaccharide and cross-linker, as described in Table 6.1.

| [UL] (% w/v) | XL:UL (weight ratio) | Compressive Modulus (MPa) | 24h Water Uptake (%) | Approximate Structural Stability |
|---|---|---|---|---|
| **4** | 0.20:1.00 | Readily soluble in aqueous media | | |
| | 0.27:1.00 | | | |
| | 0.53:1.00 | | | |
| **5** | 0.14:1.00 | 0.56±0.21 | 809.60±291.35 | Up to 3 days |
| | 1.50:1.00 | 0.23±0.04 | 1756.32±93.21 | Up to 7 days |
| | 2.00:1.00 | 0.51±0.11 | 1482.64±103.03 | Up to 7 days |
| **6** | 0.13:1.00 | Readily soluble in aqueous media | | |
| | 0.18:1.00 | | | |
| | 0.36:1.00 | | | |
| | 0.57:1.00 | 0.39±0.11 | 1282.20±158.88 | Up to 3 days |
| **8** | 0.25:1.00 | 1.44±0.34 | 1404.57±271.78 | Up to 7 days |
| | 0.50:1.00 | 3.60±0.55 | 1621.35±130.44 | Up to 7 days |
| | 0.75:1.00 | 2.60±0.61 | 1342.29±82.29 | Up to 7 days |
| | 2.00:1.00 | 1.52±0.29 | 2000.99±119.01 | Up to 7 days |

The analysis of Table 6.2 demonstrates that both cross-linker and polysaccharide concentrations markedly influence the final properties of the produced structures, in particular their compressive modulus and water uptake ability. In a first analysis, low

concentration of polysaccharide and/or cross-linker yield water soluble or rapidly degrading structures. On the other hand, increasing concentrations of ulvan appear to favour an improvement of mechanical performance of the 3D structures. However, regardless of polysaccharide concentration, higher cross-linker concentrations may result in a negative effect over this property, as it can be seen for ulvan 8% (w/v) with a 2.00:1.00 (XL:UL) cross-linker ratio.

Overall, these results indicate that it is possible to manufacture different structures with remarkably different characteristics that can be tuned in order to obtain an optimum ratio between functionality and applicability. Presented ulvan structures have a compressive modulus that can vary from 0.23 to 3.60MPa, a water uptake ability ranging from about 800 to nearly 2000% that can be readily soluble in aqueous media or present varying dissolution times within a range of 3 to 7 days.

In order to better understand the properties of the produced cross-linked ulvan structures and the effect of polysaccharide concentration, further structural characterization was performed for two conditions featuring the same cross-linker amount – 2.00:1.00 (XL:UL) – and different polysaccharide concentrations, namely ulvan 5 and 8% (w/v), further referred respectively as UL5 and UL8 conditions or samples. This yielded two structures with different mechanical properties (0.51±0.11 and 1.52±0.29 MPa, respectively) and water uptake ability (1482.64±103.03 and 2000.99±119.01 %, respectively), with similar stability in PBS (up to 7 days).

Swelling and weight loss ratios of the produced structures are represented in Figures 6.2 and 6.3. Both structures demonstrate a remarkable uptake of the surrounding fluid since the initial time point and this ability is maintained throughout the time. However, it is more pronounced in UL8 when compared to UL5. In UL8, water uptake increases until day 5; on the other hand, after one day of immersion in PBS, the water uptake ability of UL5 stabilizes. Both these structures are prone to hydrolytic degradation, as demonstrated in Figure 6.3. After one day of soaking, significant weight loss is observed (10 to 15% in mass). UL8 remains stable until the end of the experiment. However, after three days of immersion in PBS, a dramatic increase in weight loss occurred in UL5. After five days, this structure revealed signs of significant degradation, being to fragile too be handled in the last time point.

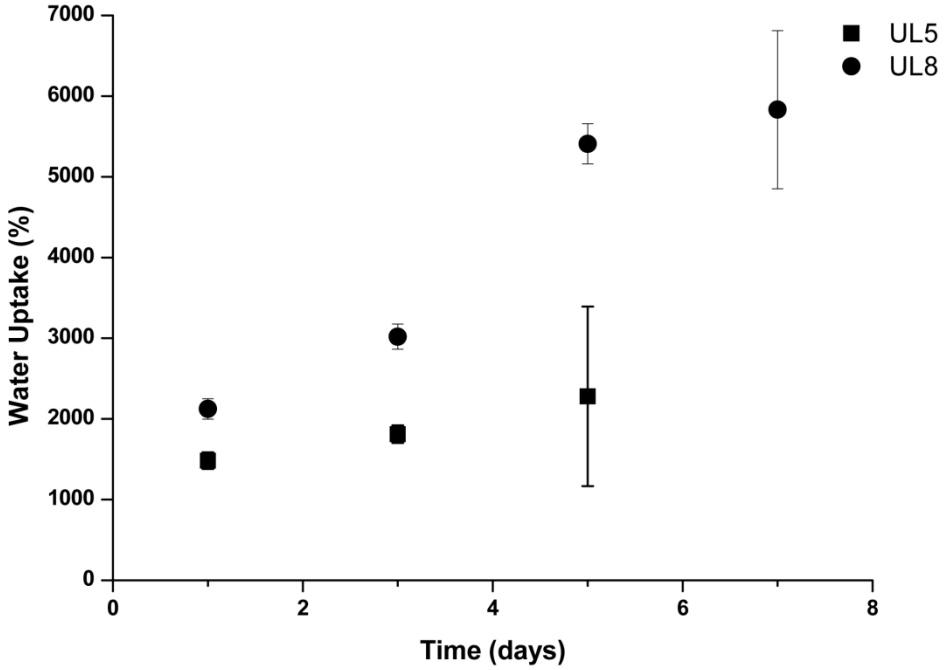


**Figure 6. 2.** Swelling ratio of produced ulvan structures (UL5 and UL8) in PBS as a function of time.

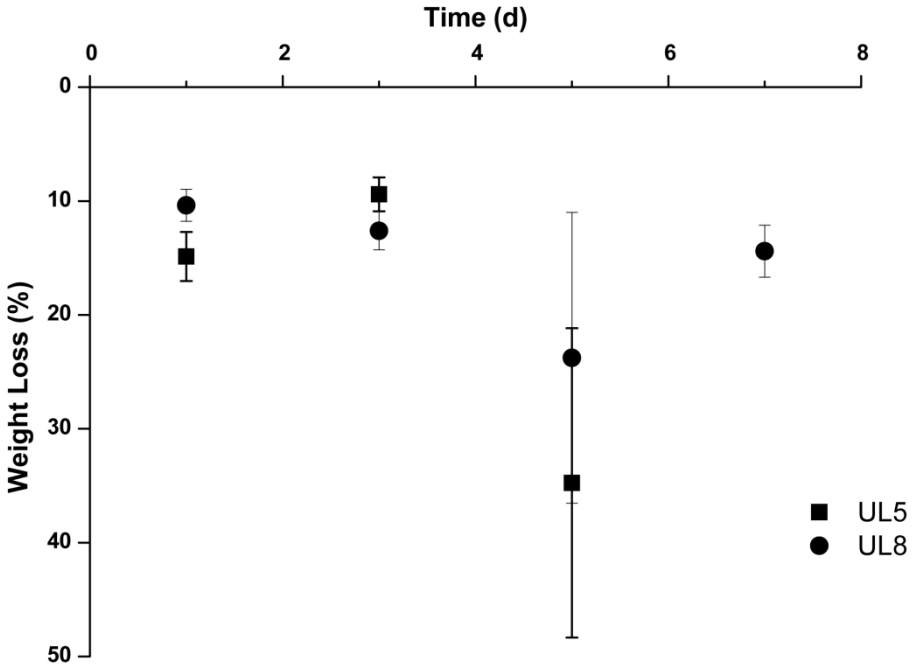


**Figure 6. 3.** Weight loss of produced ulvan structures (UL5 and UL8) in PBS as a function of time.

Morphology and pore structure and distribution of a scaffold envisaged for tissue engineering applications represent crucial parameters that allow and support cellular colonization of the polymeric matrix. In this regard, ulvan structures are highly porous,

as demonstrated by SEM micrographs (Figure 6.4) and confirmed by micro-CT analysis (Table 6.3).

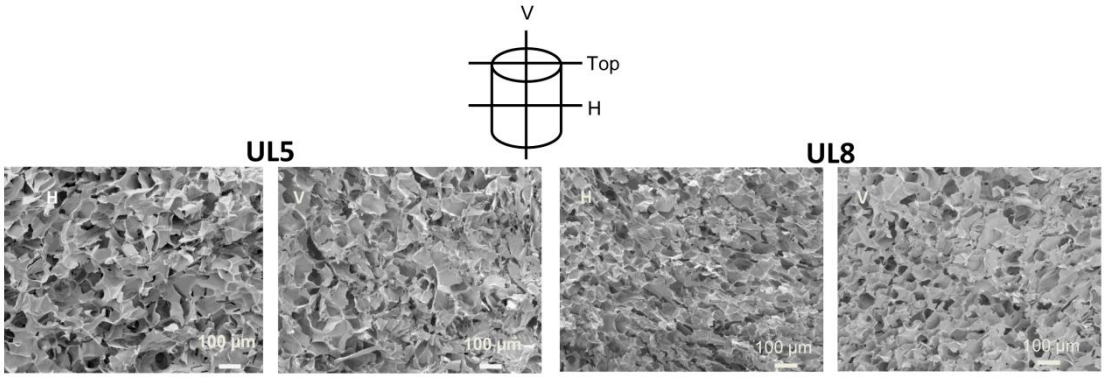


**Figure 6. 4.** Scanning electron microscopy micrographs of ulvan produced structures from different perspectives – top: top view; left: horizontal cross-section; right: vertical cross-section. (Mag. 250x).

**Table 6. 3.** Morphometric parameters characteristic of produced ulvan structures, determined by micro-CT, from analysis of UL5 and UL8 conditions. The effect of ulvan concentration on morphometric properties was analyzed by the Mann Whitney test and (*) denotes a significant difference between both structures.

| | UL5 | UL8 |
|---|---|---|
| **Porosity (%)** | 79.69 ± 0.81 | 73.10 ± 0.86(*) |
| **Mean Pore Size (µm)** | 60.40 ± 1.34 | 47.90 ± 0.89(*) |
| **Interconnectivity (>13µm) (%)** | 76.15 ± 0.95 | 67.07 ± 1.44(*) |
| **Interconnectivity (>53µm) (%)** | 19.08 ± 3.55 | 4.21 ± 0.13(*) |

For UL5 and UL8, porosity was determined to be 79.69±0.81 and 73.10±0.86% and mean pore size to be 60.40±1.34 and 47.90±0.89µm, respectively. Interconnectivity was calculated assuming two minimum pore sizes, of 13.4µm and 53.5µm for each sample. This aimed at evaluating the fraction of pores within the structure that are easily reached from the outer surface through openings of a certain minimum size.[26] In the case of UL5, interconnectivity of pores with a diameter above 13µm was calculated to be 76.15±0.95%; for pores with a diameter above 53µm interconnectivity is 19.08±3.55%. For UL8 samples, the percentage of pores with a diameter above 13 and 53µm which are interconnected was calculated to be 67.07±1.44% and 4.21±0.13%, respectively. Porosity, mean pore size and interconnectivity significantly decreased upon increase of ulvan concentration. Furthermore, this decrease in porosity correlates well with the observed differences in mechanical performance for both structures, where UL8 structure demonstrates higher compressive modulus compared with UL5.

Pore distribution is depicted in Figure 6.5 and reveals a relatively homogeneous pore size distribution, ranging from 7 to 114µm.

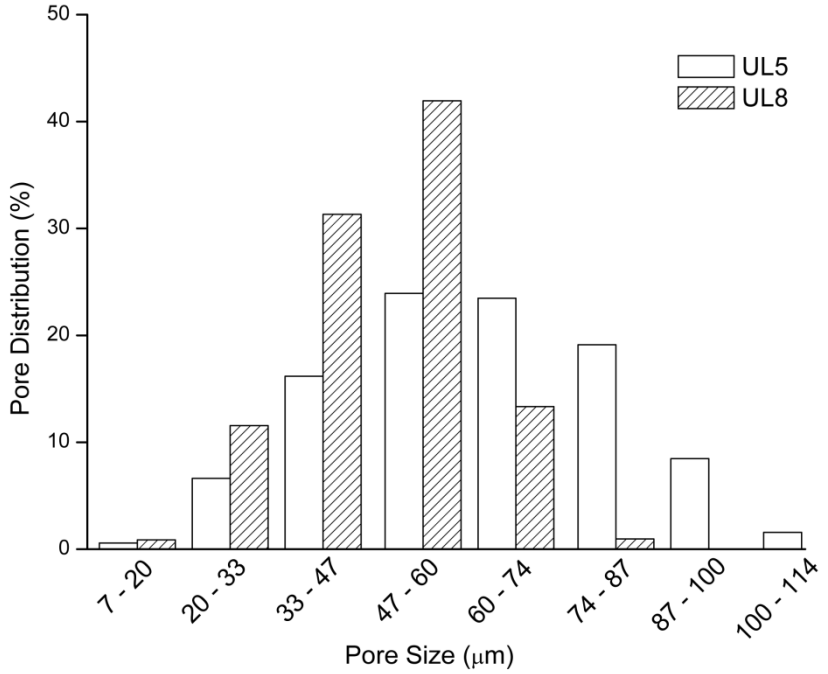


**Figure 6. 5.** Pore size distribution of UL5 and UL8 structures assessed by micro-CT analysis.

MTS assay was performed in order to evaluate cellular metabolic activity in ulvan structures, as depicted in Figure 6.6. After 1 day of culture, cells present the same metabolic activity for both ulvan structures. This activity significantly increases over time until 3 days of incubation, for both structures. However, after 3 days of incubation, L929 cells demonstrate a significant increase on metabolic activity in the scaffold with the highest concentration of ulvan (UL8) as compared with UL5. Cells cultured on both ulvan structures remained viable throughout the time of experience.

In order to correlate metabolic activity with cellular proliferation, dsDNA quantification was also performed and these results are depicted in Figure 6.7. The significant increase of metabolic activity observed after 3 days of culture for UL5 condition is associated with a significant increase in dsDNA, which can be correlated with cellular proliferation. However, and even though metabolic activity of L929 cells cultured in UL8 structure significantly increased after 3 days of culture, this is not associated with cell proliferation, as this significantly decreased.

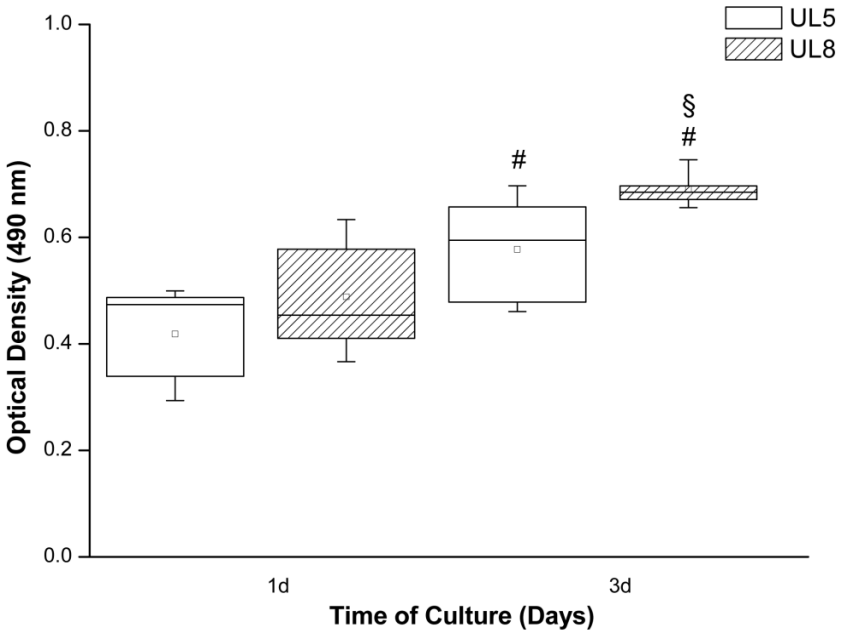


**Figure 6. 6.** Box plot of metabolic activity of L929 cells cultured on two ulvan conditions, UL5 and UL8. Data was analyzed by nonparametric Mann Whitney test: #) denotes significant differences when compared with 1 day of culture and §) denotes significant differences when compared with UL5 structure for the same time point.

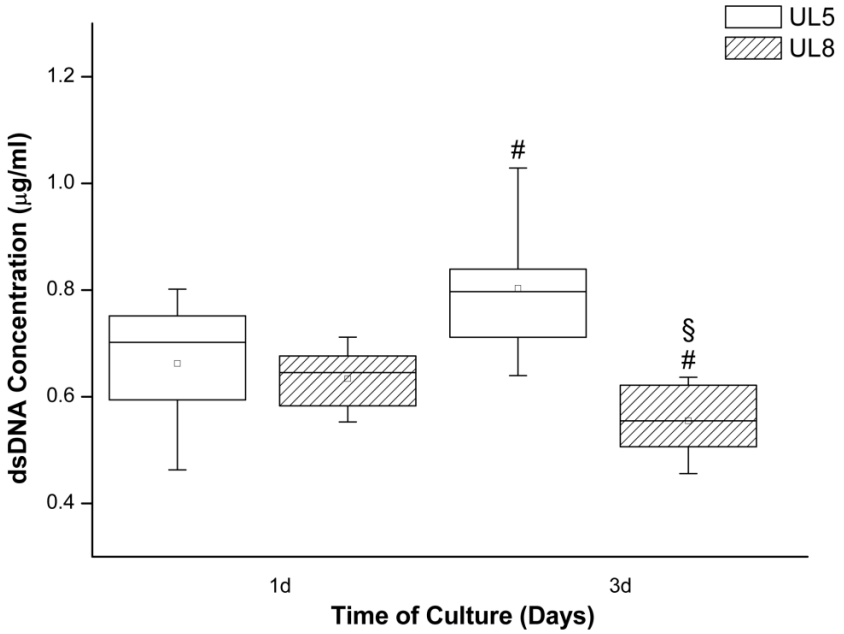


**Figure 6. 7.** Box plot of L929 dsDNA concentration cultured for two ulvan conditions, UL5 and UL8. Data were analyzed by nonparametric Mann Whitney test: #) denotes significant differences compared with 1 day of culture and §) denotes significant differences compared with UL5 structure for the same time point.

When developing degradable materials, it is important to consider leachables resulting from possible residual reagents within the polymeric matrix or from polymer degradation, which may possess inherent toxicity. In this sense, both MTS assay and dsDNA quantification were performed on L929 cells attached to the culture plate, which grow in the presence of these products, if available.

Metabolic activity of L929 cells attached to the culture plate, measured by MTS assay, is depicted in Figure 6.8. dsDNA quantification, representing cell number and growth is depicted in Figure 6.9.

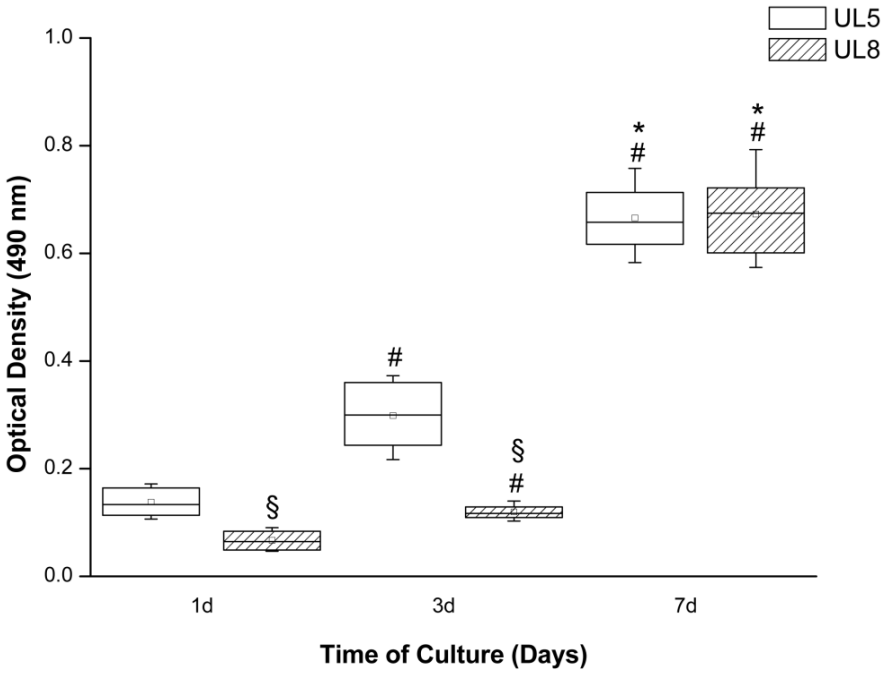


**Figure 6. 8.** Box plot of the metabolic activity of L929 cells attached to the culture plate and growing in the presence of two ulvan conditions, UL5 and UL8. Data were analyzed by nonparametric Mann Whitney and Kruskall Wallis tests: #) denotes significant differences compared with 1 day of culture, *) denotes significant differences compared with 3 day of culture and §) denotes significant differences compared with UL5 structure for the same time point.

In Figure 6.8 it is possible to observe that for the first three days, cells attached to the culture plate are viable and there are significant differences between both scaffolds, as cells growing in the presence of UL8 demonstrate lower metabolic activity when compared with UL5. In this particular context, it is possible to infer that this metabolic activity may be related to the number of cells actually attached to the bottom of the culture plate, which may be significantly different in both cases. This hypothesis is

confirmed by the analysis of Figure 6.9. The number of cells, quantified by dsDNA, for the first day of the experiment is significantly different for both structures. There are fewer cells attached to the bottom of the culture plate containing UL8 sample as compared with the one containing UL5. This fact may explain the differences observed in metabolic activity for cells growing for both conditions, even after 3 days of culture. Nevertheless, after 7 days of culture, metabolic activity and cellular proliferation of cells increased in a significant manner. After 7 days of culture, the differences observed between both samples for the first days are no longer observed. After this period, it is important to note that both ulvan structures became unstable and showed signs of considerable degradation. This suggests a remarkable increase of residual products in the cell culture media. Nevertheless, by observing MTS and dsDNA results, after 7 days, it is possible to note that cells are still viable and growing, even in the presence of these products. As a result, it is possible to conclude on the non-cytotoxic behaviour of both the samples and respective residual products.

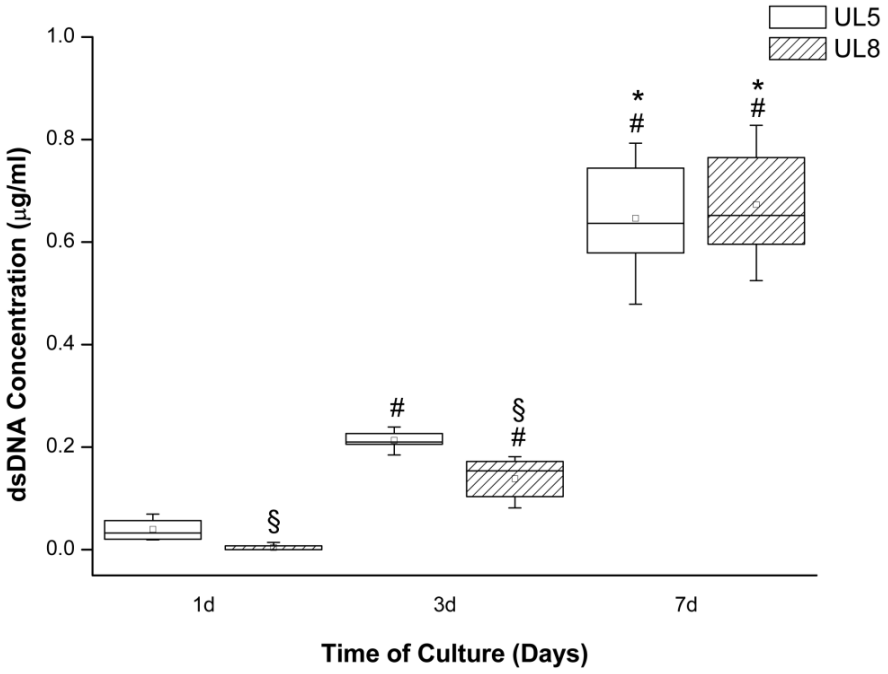


**Figure 6. 9.** Box plot of dsDNA concentration of L929 cells attached to the culture plate and growing in the presence of two ulvan conditions, UL5 and UL8. Data were analyzed by nonparametric Mann Whitney and Kruskall Wallis tests: #) denotes significant differences compared with 1 day of culture, *) denotes significant differences compared with 3 day of culture and §) denotes significant differences compared with UL5 structure for the same time point.

In order to confirm cell viability and observe cellular morphology, reflected/transmitted light microscopy, following calcein-AM and propidium iodide staining, and scanning electron microscopy were performed. As it was the case for MTS and dsDNA analysis on the polymeric matrices, this microscopic observations were only performed for the two first time points (1 and 3 days of culture), as ulvan structures were too fragile too be handled in the last time point.

Reflected/transmitted light microscopy and scanning electron microscopy images are grouped in Figure 6.10. In light microscopy observations (A) it is possible to witness the presence of viable cells for both conditions; dead cells (stained red) are almost undetected. For both cases, and as demonstrated by SEM micrographs (B), attached cells appear to retain a round morphology.

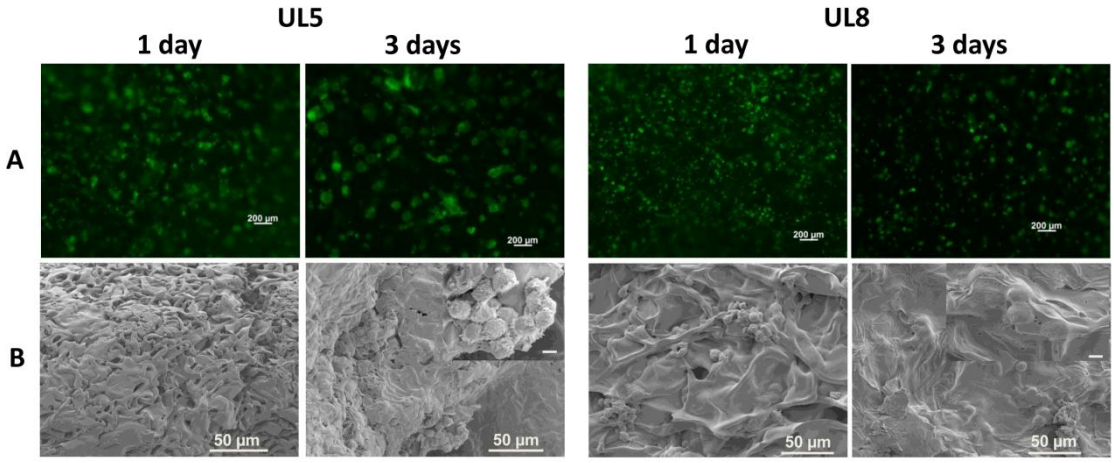


**Figure 6. 10.** Microscopic observation of L929 cells cultured in UL5 and UL8 conditions. Samples with green calcein-AM (viable cells) and red propidium iodide (dead cells) fluorescence staining, observed with reflected/transmitted light microscope after 1 and 3 days of culture. (Magnification: 5x). B: Scanning electron microscopy micrographs of UL5 and UL8 ulvan conditions cultured with L929 cells after 1 and 3 days. (Inserts: 5µm scale bar).

## 4. Discussion

Ulvan cross-linking was performed by means of a bifunctional polyexpoxide, 1,4 – butanediol diglycidyl ether (BDDE). This epoxide is able to react with different functional groups present within the polysaccharide backbone.[11-12] By varying the pH of the reaction media, different linkages are formed.[12, 21, 27] In the present research work, alkaline pH was preferred in order to try to obtain stable ether linkages. Nevertheless,

in the range of polysaccharide concentrations and cross-linker ratios investigated, maximum stability of the scaffolds upon PBS immersion was 7 days. Within this context, it is possible to infer on the low efficiency of cross-linking procedure herein applied to chemically modify ulvan and produce 3D porous structures, especially considering the stability at physiological pH of ether bonds that are expected to be formed when chemical reaction occurs in alkaline media.[12, 21, 27] However, Malson[21] has highlighted the importance of the pH of the reaction mixture immediately prior to drying as this will also influence bonds that will be formed during drying. During drying, polysaccharide chains may come closer allowing the formation of novel interconnections.[21] In this second reaction step, it is possible to hypothesize on the role played by available epoxide pendent groups introduced during the first cross-linking reaction.[25] As the first cross-linking reaction is performed in alkaline media and the pH of solution prior to drying is close to neutral, it is possible to infer the presence of a mixture of hydrolytic degradable ester and stable ether bonds in the final cross-linked structure, which may explain the degradation time observed for the produced ulvan structures. Another possible explanation to these results may lay on the polysaccharide itself, particularly its composition and conformation in solution. In fact, it has been demonstrated that ulvan adopts a closed bead-like conformation in aqueous solutions, enhanced by alkaline pH, which may limit the availability of ulvan's functional groups to chemical modification and cross-linking in aqueous alkaline media.[28] On the other hand, molecular cross-links may be impaired by highly negative sulphate groups and/or hydrophobic methyl groups ubiquitous in ulvan backbone.[29-30]

Nevertheless, *in vitro* stability will define the limits of applicability of these ulvan structures and impair their use towards applications where prolonged stability is required. In this regard, several approaches should be further investigated in order to improve stability, including an increase of the cross-linking ratio or even applying a double cross-linking technique.[12]

In the present study, different structures with different properties were produced by varying the amount of polysaccharide and cross-linker. Resulting cross-links influence the final properties of structures as reflected by the results herein obtained. Observed differences between different structures may be related with the cross-linking efficiency and/or with the concentration of the polysaccharide itself. Even though it is possible to hypothesize on the individual effects of each factor, obtained results may be a compound effect of both parameters. In fact, observed differences regarding mechanical performance, water uptake ability and weight loss may be attributed to an

increase in polysaccharide interactions that can result from increasing polysaccharide concentration or chemical cross-links.[31]

Since ulvan samples were produced applying a methodology that relies on solvent freezing, using ice crystals as porogens, resulting porous architecture is strongly influenced by solution properties as well as by molecular arrangements. In this context, the decrease in porosity, mean pore size and interconnectivity observed when polysaccharide concentration increases may be related with the increase in the volume occupied by the polysaccharide, restricting the growth of ice crystals.[32] This decrease in porosity may also contribute to the increase in the compressive modulus, observed in UL8.

Pore architecture plays a key role on the ability of a scaffold to ensure a suitable framework for cellular organization and neo-tissue formation.[2, 10, 13, 33] Produced ulvan structures are highly porous and interconnected. Although, resulting mean pore size varies from 47 to 60 µm, interconnections are in the range of 13 to 53µm, which may restrain normal cellular spreading throughout the overall structure. This observation is a possible explanation for the behaviour of cells cultured on these ulvan structures. Cells are viable in the presence of produced ulvan samples and related by-products. However, when cultured in these structures viable L929 cells present a round morphology. As cells remain viable in the presence and/or cultured in the produced structures, these observations are not due to possible cytotoxic effects of the material itself and its by-products. Incoming cells are sensitive and able to recognize the chemical composition and structural organization of the polymeric matrix.[2, 13, 34] For example, the considerable hydration of ulvan structures may hamper the ability of the scaffold to attract adhesion molecules and cells. It is generally accepted that highly hydrophilic materials when in an aqueous environment will uptake large amounts of the surrounding fluids, allowing absorption and diffusion of various solutes.[35] This considerable hydration may mask surface chemistry and incoming molecules and cells will first sense these water molecules and may not be able to recognize the appropriate material's functional groups.[2, 13, 34-36] As ulvan possesses various groups that can bind water molecules via hydrogen bonds (carboxyl, hydroxyl and sulphate groups), this may be a possible explanation for the presented results. Another explanation could be the morphology of the produced structures, which is considered paramount for cell adhesion, spreading and proliferation.[2, 10, 13, 33] Pores that are too small and reduced limits of interconnectivity, as the ones observed for ulvan samples by SEM and micro-CT, may limit cell colonization and proliferation.[9, 13, 36] These observations are particularly relevant for ulvan structures with higher polysaccharide concentration

(UL8), when compared with UL5. UL8 structures present higher compressive modulus and water uptake ability and decreased porosity and pore size. Although cells remain viable cultured on UL8, proliferation, quantified as dsDNA concentration, is not observed after 3 days of culture. UL5 structures, on the contrary, appear to support both cellular viability as well as growth.

## 5. Conclusions

The main goal of the herein described research work was to produce 3D porous structures, based on cross-linked ulvan, and evaluate their feasibility to be applied in a tissue engineering context. Produced structures present different mechanical properties and water uptake ability and are characterized by a porous architecture and undergo non-cytotoxic degradation. Increasing the concentration of ulvan revealed a striking effect both over the obtained structure and cellular behavior. Herein presented results justify further work, namely in terms of functionalization of these ulvan structures in order to optimize and attain envisaged physicochemical and biological interactions necessary to confirm the potential of ulvan for tissue engineering applications.

## Acknowledgments

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# Chapter 7

# PDLLA enriched with ulvan particles as a novel 3D porous scaffold targeted for bone engineering

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This chapter is based on the following publication: Alves A, Duarte ARC, Mano JF, Sousa RA and Reis RL, PDLLA enriched with ulvan particles as a novel 3D porous scaffold targeted for bone engineering. 2012. Submitted.

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## Abstract

A marine derived polysaccharide, ulvan, extracted from green algae, was combined with poly-D, L-lactic acid (PDLLA) in order to produce a novel scaffold for bone tissue engineering applications. Three dimensional (3D) scaffolds of PDLLA loaded with ulvan particles were originally prepared by subcritical fluid sintering with carbon dioxide at 40ºC and 50bar. Prepared matrices were characterized in order to validate their suitability to be used as scaffolds for bone tissue regeneration. Characterization included micro-computed tomography, mechanical compression testing, water uptake and degradation testing, and cytotoxicity assays. In addition, ulvan particles loaded with dexamethasone, were also dispersed within the PDLLA matrix and the respective release profile from the samples was evaluated. Prepared PDLLA scaffolds enriched with ulvan particles demonstrated appropriate physicochemical and cytocompatible features to be used for the envisaged applications. On the other hand, the release of dexamethasone from ulvan particles embedded within the PDLLA matrix revealed that the designed systems can be potentially suitable for localized drug delivery. These results further contribute to the establishment of ulvan as a potential novel biomaterial.

## 1. Introduction

Tissue engineering of musculoskeletal tissues aims to surpass their limited healing capacity. Within this context, a possible approach combines three dimensional (3D) structures with biological cues and cells to stimulate tissue regeneration while controlling cellular behavior.[1-5] This is still a rather challenging task, especially on what concerns scaffold design *per se*. An ideal scaffold must possess suitable porosity and interconnectivity, mechanical properties compliant to the site of implantation and good biocompatibility.[1-2, 6-7] Several polymeric materials have been proposed for the design of scaffolds for bone engineering, including synthetic and/or natural origin polymers.[6] These can be processed into different shapes and morphologies, according to the purpose of the application.[2, 8-9] Combination of both synthetic and natural materials is many times advantageous and broadens the range of applicability and enhances the performance of these materials.

Polyesters represent attractive synthetic materials for bone tissue engineering applications, especially poly-lactic acid and its derivative poly-D,L-lactic acid (PDLLA).[1, 6, 10] In the present research work, hydrophobic PDLLA is used as the major supporting matrix due to its appropriate degradability, compatibility and suitable mechanical performance.[11-12] In an integrated approach and in order to optimize the applicability of a PDLLA based scaffold, it is enriched with hydrophilic ulvan particles which can be used as potential vehicle for localized delivery of bioactive compounds. Ulvan is a natural polysaccharide, composed of sulphated rhamnose, xylose, glucoronic and iduronic acids, which is extracted from the cell wall of green algae *Ulva*, the common sea lettuce, which has been recently proposed for biomedical applications, with special emphasis to drug delivery.[13-14] Beyond their inherent properties, association of both these polymers would take advantage from the easiness of processing PDLLA into a three dimensional porous and stable structure, suitable for bone engineering applications, and from the feasibility of ulvan to be used as a drug delivery system.

A variety of processing techniques have been developed for the preparation of three-dimensional matrices and include solvent casting and particles leaching, compression moulding and particle leaching, thermally induced phase separation, gas-foaming processes, among others.[15-16] The main disadvantages of these methods are the use of organic solvents and the high temperatures that are often required. Furthermore, the presence of residual solvents in medical devices is restricted and ideally the levels of these residues should be minimized, if not avoided. Recently, Singh and co-workers[17] proposed a subcritical sintering method that takes advantage of carbon dioxide under subcritical conditions to prepare highly porous and interconnected structures, under

mild conditions. Carbon dioxide, under supercritical fluid conditions, is the most commonly used solvent due to its low critical parameters (T= 31ºC and P= 74bar) and to the fact that it is environmentally benign, nontoxic, non-flammable, noncorrosive, readily available and inexpensive. The subcritical sintering technique, similarly to the supercritical fluid foaming relies on the plasticizing effect of carbon dioxide which reduces the glass transition temperature of PDLLA. In the sintering process, the polymer is plasticized in mild conditions and the polymer particles are fused together, creating a 3D scaffold with appropriate morphological properties for tissue engineering and regenerative medicine.[18] This method eliminates the need of organic solvents and the high temperatures generally required to process synthetic polymers, with the additional advantage of allowing the processing of synthetic PDLLA enriched with particles loaded with labile bioactive agents.[17, 19-23] On the other hand, it has been shown[24] that the introduction of natural-based particles within a polyester matrix can enhance the application of these porous structures in bone tissue engineering applications, as they play an interesting role in the delivery of bioactive agents to signal and direct tissue growth.

In this work, the combination of ulvan, a novel hydrophilic marine origin polysaccharide loaded with a bioactive agent, with PDLLA, a hydrophobic synthetic polymer, was attempted. The effect of ulvan particles over the final structure was evaluated and the feasibility of these scaffolds focused for bone engineering applications was assessed, particularly for applications where a sustained release of dexamethasone may be desired.

## 2. Experimental procedure

### *2.1. Materials*

Ulvan was extracted and purified from green algae, namely *Ulva lactuca*, applying a procedure developed by the authors.[25] Briefly, Soxhlet extraction of dried Ulva lactuca removed most of the lipids and colouring matter. The residual off-white weed was dried and subjected to three hot-water extractions, between 75-85ºC. After filtration through a cotton cloth, aqueous extracts were centrifuged, and the liquid supernatant was filtered. The water extract was concentrated until 10-20% of its initial value, in a rotary evaporator. Starch and proteins were removed by enzymatic digestion. Afterwards, the solution was decolorized and deodorized by adsorption on activated charcoal. The water extract was centrifuged, filtered and precipitated with 4 vol. of absolute ethanol. Finally, the recovered precipitate was freeze dried. Yield of a white polysaccharide

resulting from this extraction methodology is c.a. 10 – 20%. PDLLA with an inherent viscosity of 1.87dL/g was purchased from Purasorb (The Netherlands). Carbon dioxide (99.998mol %) was supplied by Air Liquide (Portugal).

Chitosan was purchased from Sigma–Aldrich (Germany), with medium molecular weight and purified through a re-precipitation method, described by Signini and Filho.[26] Briefly, a solution of chitosan 1% (w/v) in 2% (v/v) aqueous acetic acid is prepared and filtered twice to remove any insoluble material. Chitosan is precipitated with 1M aqueous sodium hydroxide. Precipitate is recovered by filtration, extensively washed with distilled water, until a neutral pH is reached, and freeze dried. White flakes of this polysaccharide are obtained.

Unless otherwise stated, chemicals were bought from Sigma–Aldrich (Germany) and used as received.

### *2.2. Ulvan particles*

Ulvan particles were produced by extrusion - dripping method. Briefly, an aqueous ulvan solution (6% w/v) is prepared and placed in a syringe to be added in a drop wise manner to a chitosan solution (1% w/v in 1% v/v acetic acid). This procedure was performed at room temperature. Contact time of ulvan particles with chitosan solution was 2h. After stabilization, ulvan particles were collected, extensively washed and dried. For the incorporation of dexamethasone, identical conditions were applied. In this case, the drug was directly added to ulvan solution at a concentration of 15% (w/w).[27]

### *2.3. Subcritical sintering*

Scaffolds were prepared by subcritical fluid assisted sintering method at 50bar and 40ºC. Approximately 120 mg of a mixture of PDLLA and ulvan particles (non loaded or loaded with dexamethasone) were placed in a mould, which was inserted in a high pressure vessel. The vessel was heated by means of an electric thin band heater connected to a temperature controller. The sintering occurred within 30 minutes, which was the optimum contact time to allow the plasticization of the polyester. After this, the system was quickly depressurized.

### *2.4. Micro-computed tomography (μ-CT)*

Scaffolds herein described were analyzed by micro-computerized tomography using a high-resolution micro-CT SkyScan 1072 scanner (Skyscan, Belgium) to characterize its morphological and morphometric properties. The X-ray source was set at 54kV of energy and 183μA of current, Resolution of pixel size was set at 14.71μm and an

exposure time of 1.6sec was used. Approximately 450 projections were acquired with a rotation range of 180º and, using a rotation step of 0.45º. After reconstruction (NRecon, SkyScan, Belgium) of the acquired data, images of 1024x1024 bitmap were obtained and representative binary images of approximately 175 slices with a dynamic threshold of 50–255 (grey values) were generated and analyzed (CT Analyser®, SkyScan, Belgium). Different morphometric parameters were determined, including porosity, mean pore size and respective distribution and scaffold interconnectivity. This last parameter was calculated assuming a minimum pore size of 59µm for interconnected pores; that is to say that interconnection diameters lower than this value were not considered as open pores. In this sense, interconnectivity of the scaffold is calculated according to the formula:

$$I = \left[\left(V_{totalpore} - V_{disconnectedpore}\right) / V_{totalpore}\right] \times 100$$

where the volume of the disconnected pore stands for the disconnected pore volume which was defined to be higher than 59µm.

### *2.5. Mechanical compression tests*

Compressive mechanical properties of the prepared scaffolds (cut into a final cylindrical shape with 5mmx5mm) were measured using an Instron 5543 (Instron Int. Ltd., UK) universal testing machine with a load cell of 1kN. Compression testing was carried out at a crosshead of 2mm/min, until a reduction in samples height of 60% was attained. Compressive modulus is defined as the slope of the straight line obtained by linear regression of the stress-strain curve in the near elastic region of the material (between 0 and 1.0 % strain). Presented data is the result of the average of at least five measurements.

### *2.6. Water uptake and degradation tests*

Samples were weighted out into screw-top plastic tubes. The samples were immersed in 5 mL phosphate buffered saline (PBS) and allowed to swell at 37ºC, under gentle agitation (60rpm) in a precision water bath. After each time point (1, 3, 7, 14 and 21 days), the samples were brought to surface dryness, on a Whatman filter, for 10s, and weighted.

The content of water in the swollen scaffolds was calculated by the following equation:

$$Water\ Uptake\ (\%) = [(Ws - Wi)/Wi] \times 100$$

where $w_i$ is the initial weight of the specimen before immersion and $w_s$ is the weight of the swollen structure.

After each time period, the samples were dried and weighted to determine the weight loss, which was calculated according to the equation:

$$Weight\ Loss\ (\%)\ = [(Wd - Wi)/Wi] \times 100$$

where $w_d$ is the final weight of the sample (dried after immersion) and $w_i$ is the initial weight of the sample.

Presented data is the result of the average of at least three measurements.

### *2.7. In vitro drug release profile*

Four specimens of the prepared samples were immersed in 5 ml PBS (pH 7.4) and incubated at 37°C for a period of 21 days. The release of dexamethasone was periodically monitored by extracting 500 µl aliquots. Retrieved aliquots were replenished with 500µl of PBS. The concentration of dexamethasone was determined by UV–Vis spectroscopy at 242nm (Shimadzu UV 1601, Japan). Presented results are an average of four measurements for each time point condition.

### *2.8. Cytotoxicity studies*

In order to assess the cytotoxicity of the obtained scaffolds, extracts of the materials were prepared and placed in contact with mouse C3H/An connective tissue fibroblast-like cells (L929) (European Collection of Cell Culture, UK). Cell viability was evaluated by MTS assay in accordance to ISO/EN 10993 part 5 guidelines.[28]

In summary, $5x10^3$ cells.ml$^{-1}$ were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Alfagene, USA) and 1% antibiotic/antimycotic solution (Gibco, UK) and allowed to grow until a desirable confluency was reached. At this time, culture media was replaced with material extracts, prepared in culture media. L929 cells viability was determined for each extract and compared to latex extracts, used as a positive control of cell death, and to cells growing in the absence material extracts (negative control, representing cell growth in culture media). After each time point (24, 48 and 72 h of incubation) MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) test was performed to assess cellular viability, following the manufacturer's instructions (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega, USA). Optical density was measured at 490nm, on a multi-well microplate reader (Synergy HT, Bio-Tek Instruments, USA). All cytotoxicity screening tests were performed on 15 replicates.

### *2.9. Statistical analysis*

Statistical analysis was performed using the GraphPad Prism statistic software (Release version 5 for Windows). Initial statistical results indicated that nonparametric tests should be used for all comparisons. Therefore, the effect of ulvan particles on mechanical performance was analysed by the Mann Whitney test. Furthermore, the effect of the produced structures over L929 viability was evaluated by Kruskal–Wallis test, followed by Dunn's test for multiple comparisons. Statistical significance was defined as $p<0.05$.

## 3. Results and Discussion

A marine derived polysaccharide – ulvan - extracted from green algae was processed into particles by adding an ulvan solution, in a drop wise manner, to a solution composed of chitosan (Figure 7.1).

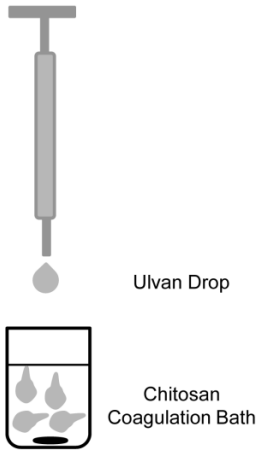


**Figure 7. 1.** Schematic representation of the method used to produce ulvan particles by addition of an ulvan solution in a drop wise manner to a coagulation bath composed of chitosan solution.

Particles are formed by the static attraction between the opposite charged polysaccharides, ulvan and chitosan. In this way, ulvan, an anionic polysaccharide, reacts with the cationic chitosan to form a well defined polyelectrolyte complex. In this scenario, the main purpose of the polycation is to induce the formation of a strong outer layer which stabilizes and strengthens the polyanion network. Consequently, the processed particles are stabilized by these electrostatic interactions formed between both polymers.[29-30] Complexed particles of ulvan/chitosan present a homogeneous

distribution of size, are hollow and exhibit well defined teardrop morphology (Figure 7.2).

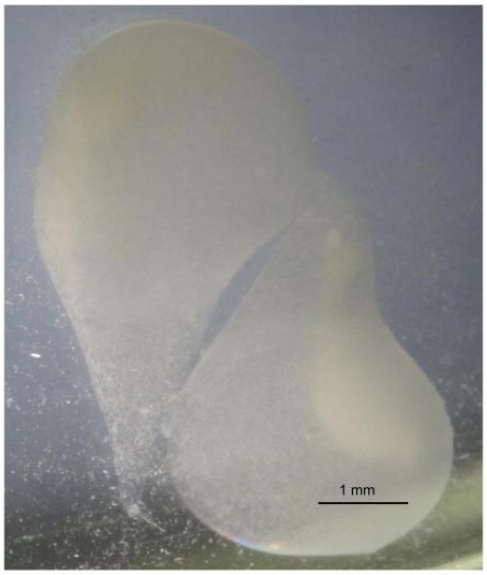


**Figure 7. 2.** Ulvan particles loaded with dexamethasone (UL+DX), visualized with a magnifying lens, presenting a tear-drop like morphology: Diameter ~3mm, height ~4.5 – 5mm.

Produced ulvan particles were combined with PDLLA in order to produce a novel scaffold, processed by subcritical sintering in the presence of subcritical carbon dioxide, targeted for bone tissue engineering. This is a rather challenging field of research. In this context, several requirements must be obeyed by the scaffold to be used in order to achieve ideal regeneration of bone, including morphological and mechanical properties, biodegradability and biocompatibility.[7]

### *3.1. Morphological Characterization*

Through modern imaging techniques, such as micro-computed tomography (micro-CT), which has already proven to be a quite powerful in scaffold characterization[31], it is possible to obtain and analyse detailed 3D reconstructions of the matrix structure. Produced scaffolds of PDLLA enriched with ulvan particles (PDLLA+UL) present a well defined porous morphology. The introduction of ulvan particles within the PDLLA matrix had a clear effect on scaffolds' morphometric properties as it is demonstrated by comparison with PDLLA scaffolds without ulvan particles (Table 7.1). Numerical comparisons of different parameters demonstrate that the presence of ulvan particles within the PDLLA matrix lead to a significant increase in the overall porosity (from 45.8±6.0 to 75.7±1.2 %), mean pore size (from 601.3±84.7 to 1005.5±74.0 μm) and interconnectivity (29.8±9.4 to 48.7±8.5 %) (Table 7.1). Produced PDDLA and

PDLLA+UL scaffolds present a heterogeneous pore size distribution ranging from 30 to 1600µm (Figure 7.3). An ideal scaffold for bone engineering should be porous, interconnected and rich in macropores.[12, 31-32] Random, open and interconnected pores favors cell penetration and nutrient exchange into the scaffold preventing the rapid formation of tissue on the surface, which would ultimately result in a necrotic core.[33]

**Table 7. 1.** Morphometric parameters characteristic of produced scaffolds determined by micro-CT analysis of PDLLA and PDLLA+UL scaffolds.

| | **PDLLA** | **PDLLA+UL** |
|---|---|---|
| **Porosity (%)** | 45.8±6.0 | 75.7±1.2 |
| **Mean Pore Size (µm)** | 601.3±84.7 | 1005.5±74.0 |
| **Interconnectivity (%)** | 29.8±9.4 | 48.7±8.5 |

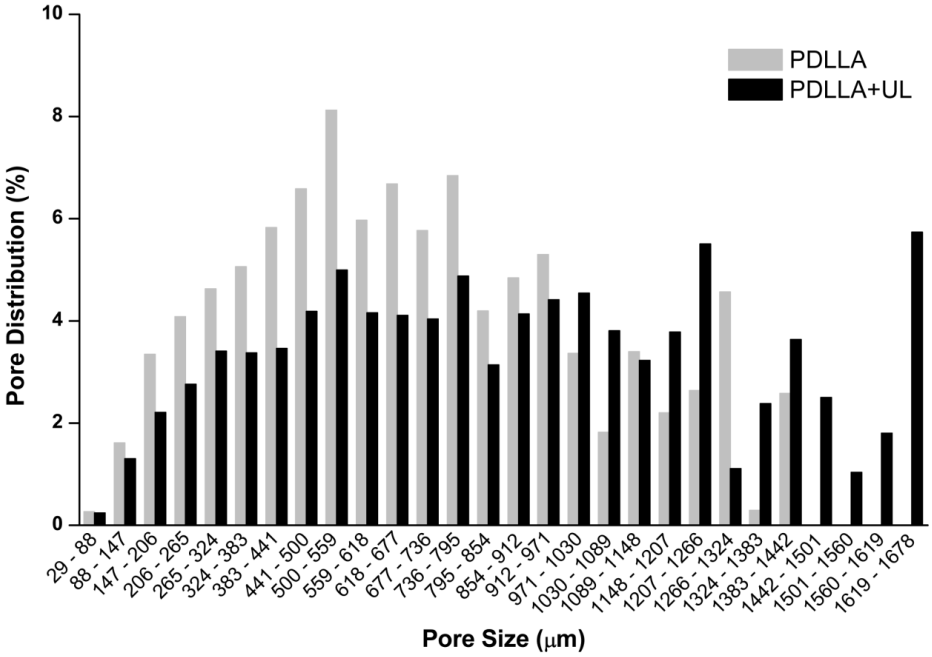


**Figure 7. 3.** Pore size distribution of PDLLA and PDLLA/Ulvan scaffolds prepared by subcritical sintering and assessed by micro-CT analysis.

### *3.2. Mechanical Properties*

Besides the morphological aspects, mechanical performance of the matrices is one of the key issues when designing scaffolds for tissue engineering applications. Bone possesses remarkable mechanical properties, which are difficult to mimic. It is a complex structure which requires mechanical stimuli to induce tissue growth, remodeling and regeneration.[7, 34] In this sense, mechanical properties assume a crucial role in scaffold design for bone engineering. In fact, an implant with higher mechanical

properties will absorb the entire load, causing a stress shielding effect and resulting in bone resorption around the implanted scaffold.[7] So, a material should mimic the mechanical properties of the tissue that is being engineered.

In the present study, enrichment of PDDLA scaffolds with ulvan particles resulted in a significant decrease of the compressive modulus, as compared with the compressive modulus of the PDLLA structure without ulvan particles (Table 7.2). This negative effect on the mechanical performance of PDLLA+UL structures can be related with observed changes on morphological properties of structures enriched with ulvan particles, namely the increase in porosity and mean pore size.[32]

**Table 7. 2.** Mechanical properties, given as compressive modulus (defined as the slope of the straight line obtained by linear regression of the stress-strain curve in the near elastic region of the material, between 0 and 1.0 % strain), of the prepared scaffolds: PDLLA and PDLLA+UL. The effect of ulvan particles on mechanical performance was analysed by the Mann Whitney test and (*) denotes a significant difference.

| | **Compressive Modulus (MPa)** |
|---|---|
| **PDLLA** | 14.4±2.9 |
| **PDLLA+UL** | 8.3±1.5$^{(*)}$ |

*3.3. Degradation*

Ideally, in tissue engineering, a scaffold is usually intended to temporary fill a defect, while gradually degrading as neo-tissue is formed. In due course, the scaffold is replaced by new bone tissue.[7] After implantation, the scaffold interacts with the tissue fluids, uptaking them at some extent, starting the degradation process.[35] Degradation of biodegradable polymers occurs as a result of different biological processes, including enzymatic reactions or passive hydrolytic degradation, among other mechanisms.[36]

In order to try to understand the swelling ability and hydrolytic degradation of the produced scaffolds, as well as the influence of ulvan particles on both these parameters, water uptake and weight loss studies were performed for different time periods for samples immersed in PBS solution (Figures 7.4 and 7.5). Although there is a higher initial increase in swelling for PDLLA+UL scaffolds as compared with PDLLA, the overall swelling is maintained over the course of time (21 days) (Figure 7.4). PDDLA is slowly degraded by hydrolytic reactions at physiological temperature.[11-12] This is demonstrated by the experimental results, as the weight loss of PDLLA

scaffolds is close to 0.5% and it maintains this behaviour over 21 days (Figure 7.5). The introduction of ulvan particles increased weight loss by 5%. This may be a net effect related with degradation of ulvan particles and increased porosity of the PDLLA+UL structure. Nevertheless, the overall process remains slow and the scaffold revealed to be very stable.

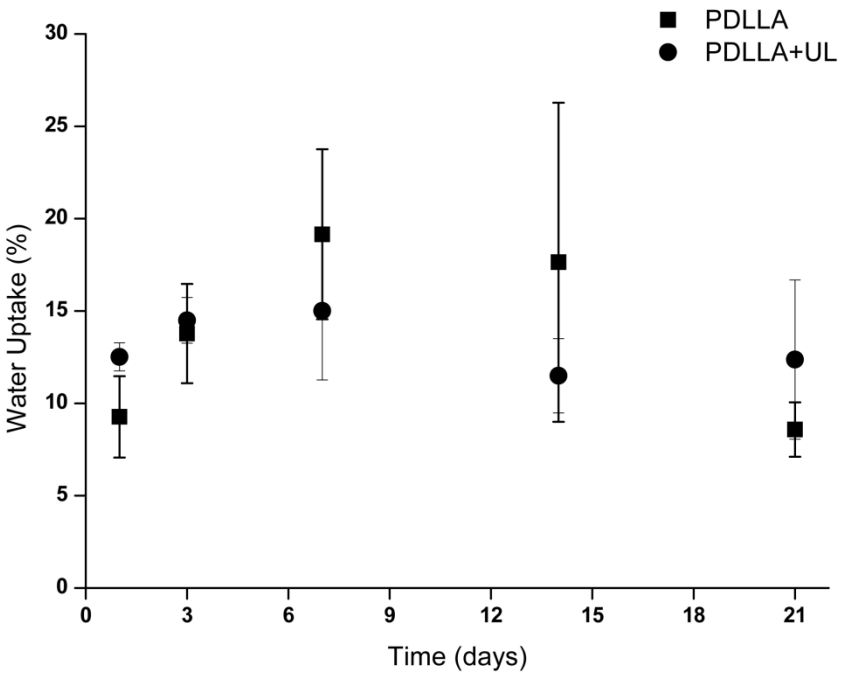


**Figure 7. 4.** Water uptake profile of PDLLA and PDLLA/Ulvan porous scaffolds immersed in PBS solution.

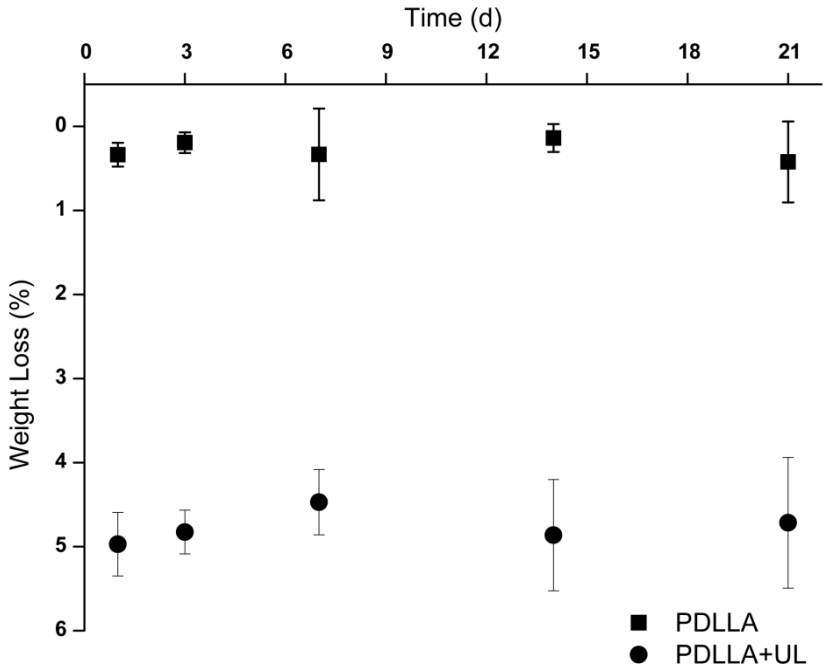


**Figure 7. 5.** Weight loss profile of PDLLA and PDLLA/Ulvan porous scaffolds immersed in PBS solution.

### *3.4. PDDLA/Ulvan scaffold for local drug delivery*

The release of dexamethasone from PDLLA scaffolds enriched with ulvan particles loaded with dexamethasone (PDLLA+UL+DX) and ulvan particles (UL+DX) was evaluated *in vitro* and the profile of drug release is presented in Figures 7.6 and 7.7.

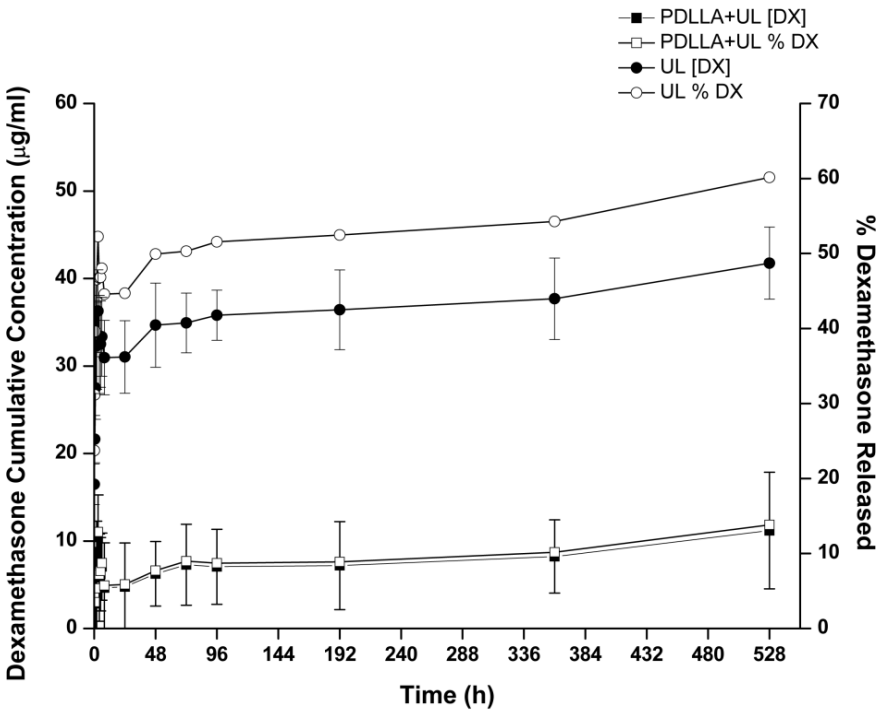


**Figure 7. 6.** Dexamethasone concentration and percentage of drug released from the produced scaffolds and ulvan particles.

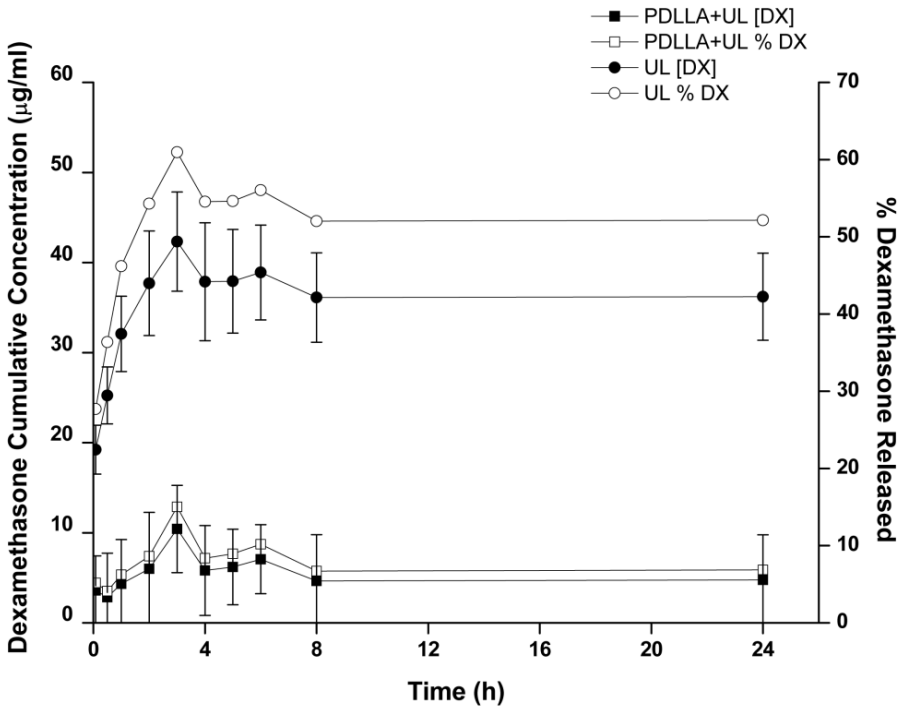


**Figure 7. 7.** Detail of dexamethasone concentration and percentage of drug released from the produced scaffolds and ulvan particles.

Ulvan particles loaded with dexamethasone demonstrate a steady release during the first 3 hours, releasing 52% of the drug. Afterwards, a slower release is observed and at day 21, a total of 60% of dexamethasone is released from this system. Incorporation of ulvan particles within a PDLLA matrix resulted in a slower release of dexamethasone, although the profile of release is maintained. In this system, after 21 days of immersion, only 13% of dexamethasone is released out of the PDLLA+UL structure. In order to understand this result, it is important to stress that different mechanisms govern the drug release rate and different phenomena might be involved in the release profile depending on the type of drug, polymer matrix, processing technique, geometry and dimensions of the carrier system.[37] The most pronounced effects are based on drug diffusion out of the release system into the release medium and water uptake ability of the matrix components.[38] The differences observed between the amount of drug released from ulvan particles *per se* and ulvan particles incorporated within the PDLLA scaffold can be attributed to these different mechanisms of drug release, particularly to the water uptake ability of the overall matrix. Water uptake measurements carried out in the present work did not reveal significant differences between the PDLLA scaffold alone and the PDLLA scaffold enriched with ulvan particles. The general swelling ability of PDLLA scaffolds is limited by the hydrophobic nature of poly-lactic acid, restraining the high swelling ability inherent to ulvan.[13] For this reason, it is possible to infer that dexamethasone release from ulvan particles entrapped within the PDLLA scaffold is governed mainly by drug diffusion out of the matrix. In the case of ulvan particles *per se*, it may be expected that they follow a case II transport as referred in a recent study for ulvan membranes. The potential of ulvan membranes to be used as drug delivery devices as been recently proposed and the analysis of their release profile indicated that the mechanism of release from this ulvan matrix falls in the case II transport. This means that the release of dexamethasone from ulvan membranes is associated with the relaxation of the polysaccharide upon hydration.[13]

### *3.5. Cytotoxicity*

Despite the morphological and physicochemical properties of a scaffold, the most important and decisive factor defining its applicability as a tissue engineering construct is its biocompatibility.[7]

In this regard, prepared scaffolds were subjected to a cytotoxicity screening. This analysis was performed by culturing cells in the presence of extracts of those materials, which may traduce potential toxicity of their leachables and degradation products, and by evaluating subsequent cell viability by MTS. This assay reveals the presence of

metabolically active cells and it is considered a realistic test to evaluate cytotoxicity. For this cytotoxicity screening, both the constituent structures (PDLLA and UL+DX) and the overall scaffolds (PDLLA+UL and PDLLA+UL+DX) were evaluated. Results are depicted in Figure 7.8.

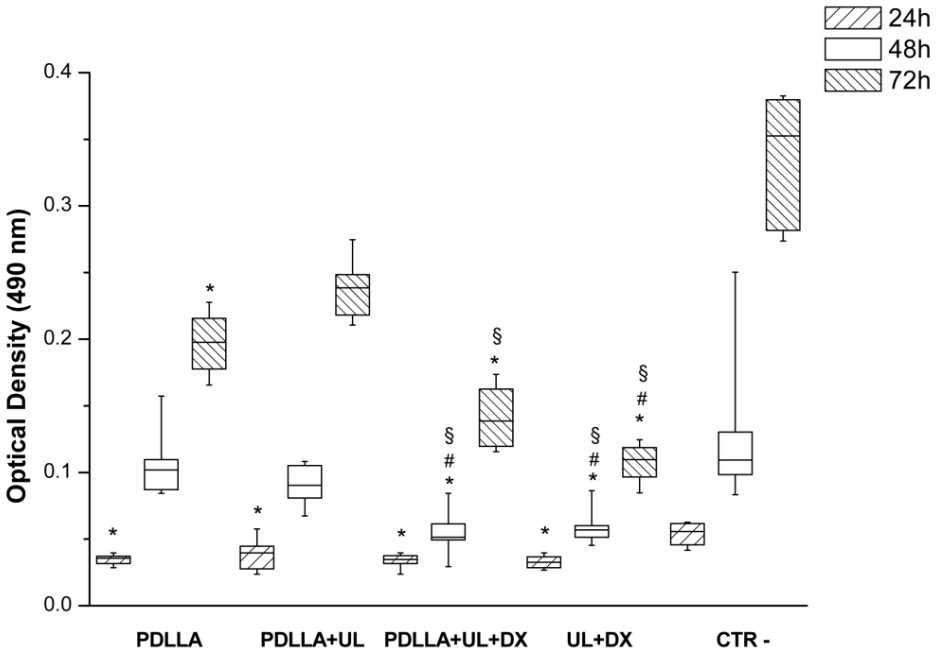


**Figure 7. 8.** Box plot of L929 metabolic activity cultured in the presence of PDLLA, PDLLA+UL, PDLLA+UL+DX and UL+DX extracts. Data were analyzed by nonparametric Kruskal–Wallis test followed by Dunn's test: *) denotes significant differences compared with negative control; #) denotes significant differences compared with PDLLA and §) denotes significant differences compared with PDLLA+UL.

In a first stage and after 24h of incubation in the presence of the different material extracts, cells demonstrate equal metabolic activity, i. e., the effect of the different materials over L929 cells is not significantly different as compared with each other. However, cells' metabolism is significantly lower than the one exhibited by the cells growing in normal conditions (negative control, CTR -). After 48 hours, this negative effect on cell viability is only observed for the structures incorporating dexamethasone (PDLLA+UL+DX and UL+DX). Cells' metabolism in the presence of these structures is affected and significant differences are observed as compared with the cells growing in the absence of material extracts and cells growing in the presence of PDLLA and PDLLA+UL. After 72h, cells cultured in the presence of PDLLA, PDLLA+UL+DX and

UL+DX extracts are significantly different from those growing in the absence of these materials. However, at this time point, cells cultured in the presence of PDLLA+UL extract demonstrate normal metabolism by comparison with cells growing in normal conditions. In spite of the differences observed by comparison with the negative control for each time point, cellular metabolism appears to be increasing over the time of incubation, for each material extract. In summary, produced PDLLA scaffold enriched with ulvan particles demonstrate good cytocompatibility after 72 hours of culture. In fact, the presence of ulvan within the polymeric matrix appears to have a positive effect on cellular viability, when compared with PDLLA alone.

## 4. Conclusions

In the present research work, a subcritical carbon dioxide sintering process was successfully employed to process a scaffold composed of PDLLA and ulvan particles. The incorporation of these particles within the PDLLA matrix resulted in a scaffold with appropriate morphometric features, with suitable mechanical performance and adequate cytocompatibility. Furthermore, entrapment of ulvan particles loaded with dexamethasone within the PDLLA matrix may enhance the applicability of this scaffold as a system able to sustain the delivery of relevant bioactive agents.

## Acknowledgments

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# SECTION V – CONCLUSIONS AND PERSPECTIVES

# Chapter 8

# Conclusions and perspectives

## Conclusions and perspectives

The main objective of the herein presented thesis was the study of the potential of ulvan, a rather unexploited polysaccharide extracted from green algae, to be used in a biomedical context. The use of ulvan as a medical device in tissue engineering and regenerative medicine applications is still in its early stages, particularly on what concerns polysaccharide modification and processing and biomaterial design. Nevertheless, it is possible to envisage the use of this polysaccharide in a biomedical area, where it can act as a support for therapy of damaged/diseased cells and tissues. On the other hand, it is known to possess a variety of distinctive biochemical properties that may enhance and define its applicability in this context.

Different industries, including biomedical and biotechnological ones, are continuously searching for new functional and better performing materials. The use of algae polysaccharides in biomedical applications is not a novelty as these polymers have been used for a long time. Agarose, carrageenan and alginates are some of the most known examples. Their uses extend from food to biomedical applications, for instance as food additives or scaffolds for cellular growth and tissue regeneration. In this context and considering ulvan's unique structure and inherent properties, it is valid to envisage ulvan as a novel polymer for potential biomedical applications.

In order to propose ulvan as a novel biomaterial, an integrated strategy should be pursued, starting with the design of a novel and effective extraction methodology followed by processing of the obtained polysaccharide into different structures that can be envisaged as potential medical devices.

In a first stage, a new and simple method of extracting ulvan from green algae was designed and the obtained polysaccharide was characterized. Developed extraction procedure has proven to be effective in yielding a non-cytotoxic polysaccharide with interesting physicochemical properties. It is a non-meltable semi-crystalline polysaccharide, highly hygroscopic and rich in sulphate groups; it is mostly composed of ulvanobiuronic acid A with minor amounts of ulvanobiuronic acid B. In general, this is a high molecular weight polysaccharide, composed of rhamnose, glucuronic acid, xylose, iduronic acid and glucose.

In order to understand the processability of ulvan extracted from green algae, different polymer processing methodologies were investigated. These included solvent casting, freeze drying and an extrusion-dripping methodology, in order to process ulvan into membranes, 3D porous structures and particles, respectively.

Ulvan membranes, designed as vehicles to deliver therapeutic agents to wounds, have been produced by solvent casting. The introduction of a bioactive agent widens the range of applicability of these membranes, as drug delivery systems, particularly as medicated wound dressings. These ulvan membranes present interesting mechanical properties, but their most distinctive feature is related with water uptake ability, which can reach values as high as 1800%. This ability demonstrates a strong influence in the delivery of a drug from these ulvan membranes; in fact, the mechanism that drives the release of the drug from these membranes is mostly related with the swelling of the polymeric matrix. As swelling is a property related with the polymer itself, it may be tuned to introduce further control to the release of the bioactive agent, if desired. Although a biocompatibility study should still be performed, these properties suggest that this natural sulphated polysaccharide can be proposed for the envisaged applications as a wound dressing, in order to provide control of wound surface hydration, absorbing excess exudates and assuring proper moisture, while delivering appropriate therapeutic agents to assist the healing process.

As part of the design of ulvan membranes, successful chemical cross-linking of ulvan was achieved with the epoxide 1,4 – butanediol diglycidyl ether (BDDE). The knowledge gathered on ulvan's chemical cross-linking was applied in the production of freeze dried 3D ulvan porous structures. Different formulations were attempted, with varying polysaccharide concentration and cross-linker ratio. Resulting structures demonstrated diverse compressive modulus and water uptake ability, as well as varying stability. This indicates that these properties can be tuned, by means of varying processing conditions, in order to obtain suitable structures for the envisaged applications. Among these matrices, two were further characterized and revealed a micro-porous, highly interconnected architecture and undergo non-cytotoxic degradation. For a tissue engineering approach, further work needs to be performed in order to assure the desired physicochemical and biological interaction.

Confirming ulvan's processing versatility, it was also processed into particles, by an extrusion-dripping method; these particles were then incorporated within a poly-DL-lactic acid matrix. Polyesters like poly-DL-lactic acid are widely used for biomedical applications and the incorporation of ulvan particles within a PDLLA matrix resulted in a scaffold with enhanced properties. The introduction of ulvan particles resulted in an increase of the overall porosity, pore size and interconnectivity. However, the presence of these ulvan structures induced a decrease in the compressive modulus of the structure, which can be related with the increase in porosity and mean pore size observed after the introduction of ulvan particles within the PDLLA matrix. Besides the

overall effect over the properties of the polyester matrix with the introduction of ulvan particles as such, the functionality of this novel scaffold can be increased with the effective introduction of an active agent. In this regard, ulvan particles embedded within a PDLLA matrix can also function as a delivery system, widening the applicability of this scaffold.

In spite of the work herein described and others reported in the literature, the study of the potential of ulvan to be used in a biomedical context, as a medical device, is still in its early stages. The herein described ulvan processing techniques and envisaged applications pose an innovative attempt to add value to this marine derived polysaccharide, aiming to expand the knowledge on novel applications of ulvan. Although the major focus of the present thesis was towards the study of potential application in a biomedical context of a polysaccharide extracted from green algae, the reality is that there is still a lot to be done at the fundamental level to clarify the knowledge about ulvan. If we compare the evolution of this polysaccharide with other algae origin polysaccharides, it has been remarkably slow. At this point, it is possible to speculate on the influence of ulvan's peculiar chemical composition and structure, complicated by the fact that there isn't yet a standardized commercial form of this polysaccharide. In this regard, the design of a novel and effective extraction procedure becomes crucial, but also opens the way to the possibility of scaling up. This is an important consideration, if continuous research on ulvan is intended. Furthermore and as greatly discussed throughout this thesis, ulvan has a peculiar structure; it is composed of rare sugars, scarcely found in algae and it is highly sulphated. This confers various properties to this polysaccharide that are still not completely studied, and both properties and this particular chemical composition and structure are still being researched, side by side with the advent of its potential development into medical devices to be applied in a biomedical context.

The work performed and described throughout the present thesis demonstrates the feasibility to extract ulvan from green algae and process it into different structures envisaged for biomedical applications. The exploitation of this polysaccharide in this context and considering the diversity of developed structures may lead to novel research opportunities. This can be considered an open field of research where novel and different polysaccharide modifications may be studied, including chemical modifications as the ones described throughout this work. Furthermore and given the processing versatility demonstrated by ulvan, novel design methodologies should be followed in order to maximize the production of optimized ulvan structures. Finally and in order to increase ulvan-cellular interactivity, studies should be performed, for

instance, on surface modification of produced structures through chemical or physical approaches. Nevertheless, the present thesis may be positioned as a transition between theoretical considerations towards a pratical knowledge leading to realistic solutions based on ulvan for specific therapeutic problems.

Despite the fact that this polysaccharide is known and being researched for several decades, its knowledge and evolution is still quite constrained. The author would like to think that this scenario is shifting and hopefully the present thesis can contribute to a better understanding of ulvan and its processability and serve as a stepping stone to future work on ulvan's applicability, especially in a biomedical highly demanding arena.